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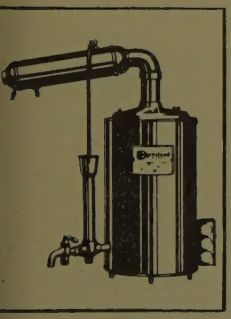

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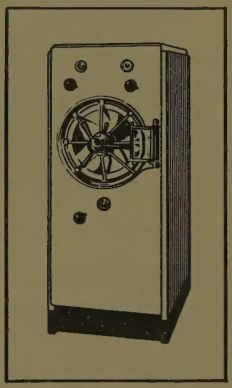

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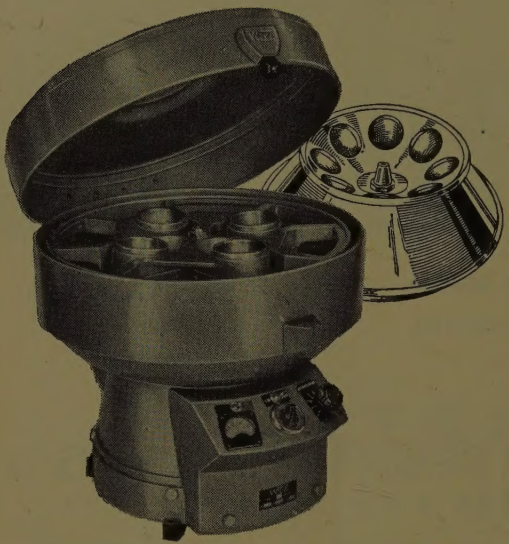

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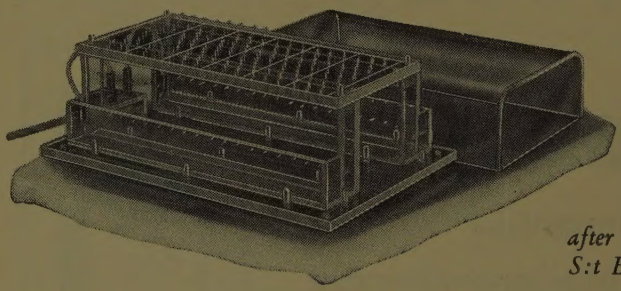

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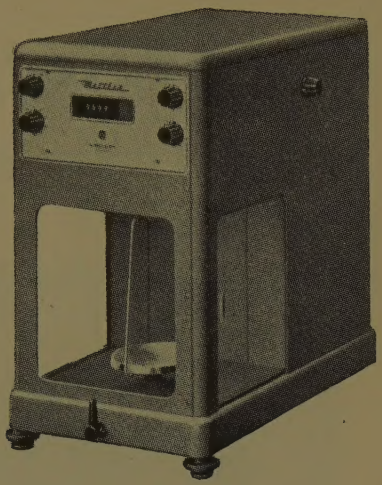

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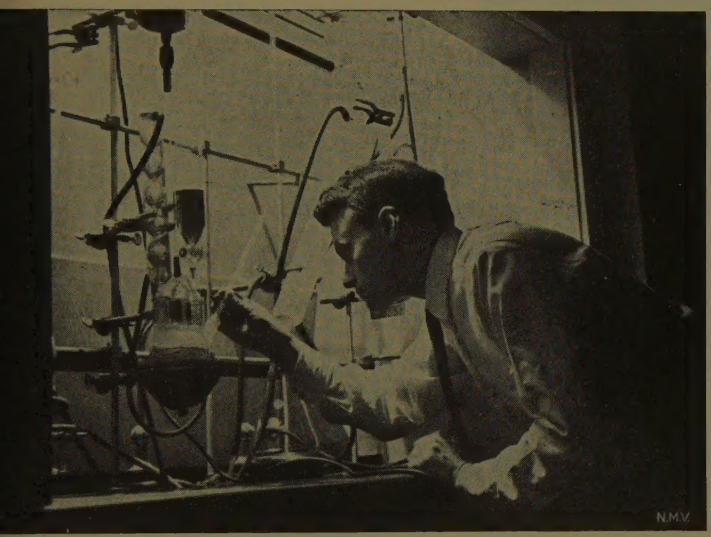

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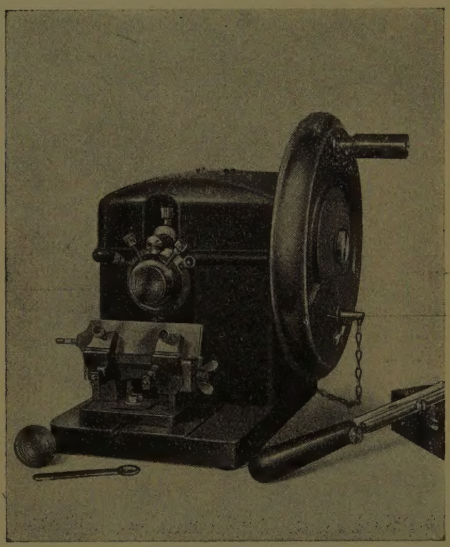

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VARIATION IN THE CHOLINESTERASE-INHIBITING EFFECT OF SUCCINYLCHOLINE

By J. STOVNER

m the Department of Anesthesia and the Central Laboratory, Rikshospitalet, Oslo, Norway

(Received for publication Jan. 25, 1955)

cinylcholine (Sch.) has a wide clinical
s a muscle-relaxant. An important
l advantage of Sch. is its relatively
inactivation in the body due to its
atic breakedown by the serum
esterase (Glick, 1941; Bovet-Nitit,
Castillo & de Beer, 1950). This
ns its short duration of action and
of cumulative effect, both desirable
ties in a muscle-relaxant.

serum cholinesterase is usually
red by the rate at which it hydrolyses
choline (Ach.) *in vitro,* either by the
pment of CO_2 in Warburg apparatus
stinsson, 1948) or by the change in
bserved by the electrometric method
el, 1949).

ns, Gray, Lehman & Silk (1953)
fairly good correlation between the
of serum cholinesterase and the dura-
f response to injected Sch. in various
ts. There are, however, cases reported
this correlation fails clinically
(Evans, 1952; Stovner, 1954). Furthermore, different mammalian species have quite different levels of serum cholinesterase (Augustinsson, 1948). There is, however, no absolute relation between the duration of response to injected Sch. and the level of serum cholinesterase in these various mammalian species.

Foldes (1954) in studying the rate of hydrolysis of acetylcholine, benzoylcholine and succinlylcholine in different human plasmas and the plasmas of various mammalian species, found no parallel between the hydrolysis rates of these various choline esters. He explains this by assuming that there is either more than one esterase capable of hydrolysing Ach. in mammalian plasma, or that qualitative differences exist in the serum cholinesterase of different individuals and different species.

The *in vitro* determination of the hydrolysis rate of Sch. by an esterase is complicated, however, by the fact that Sch.

is a diester and the number of equivalents of acid set free is not necessarily proportional to the quantity of Sch. broken down. Different esterases will act differently toward the two ester-linkages in Sch. The serum cholinesterase, for example, will fairly rapidly split off one ester-linkage in Sch., so that succinylmonocholine is formed, but this in turn is split very slowly and will accumulate (Foldes, 1953). The "true" esterase of the erythrocytes will split Sch. rather slowly, but the monoester is split almost as fast as Ach. by this esterase (Lehman & Silk, 1953).

The determination of the hydrolysis rate of Sch. by serum cholinesterase is therefore complicated by the different behaviour of the two ester-linkages in Sch. It is, however, possible to get an indication of the affinity of serum cholinesterase to Sch. by determining the inhibition caused by Sch. of the activity of serum cholinesterase. This inhibition has been determined by Tammelin (1953) using the electrometric method. He does not state the source of the serum used. In the literature there are no reports of whether this inhibition is the same in man as in other mammalian species.

If the enzyme in serum responsible for the Ach. — hydrolysis is identical with the Sch. splitting enzyme, one would expect this inhibition to be constant as expressed by the I_{50} value below. On the other hand, if there are qualitative differences, or several enzymes are responsible, the inhibition might not be constant.

The present work is an attempt to determine if this inhibition is constant in various human individuals and for different mammalian species.

METHOD

The electrometric method has been use makes use of the fact that the acetic acid pro by the hydrolysis of Ach. leads to meas changes in pH in a diemal-buffer solution. I registered the pH every 5 min. over a peri 20 min. using the glass-electrode (Radiomet Copenhagen). The pH change caused b simultaneous hydrolysis of Sch. without Ach minimal and could be neglected. However, a test with Sch. alone has always been perf and the results subtracted from the results the mixed solution of Ach. and Sch.

PROCEDURE

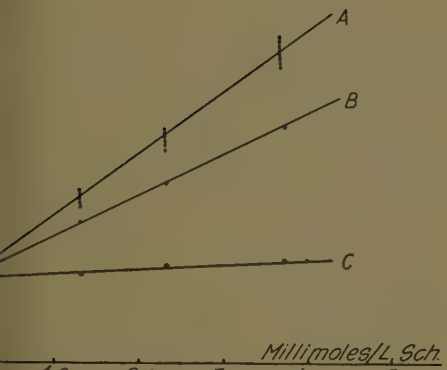
Michaelis' buffer 3 ml + plasma 0.5 ml d to twice its volume was set up. To this was 2 ml of water or 2 ml of a solution containi various amounts of Sch. This was allow incubate at room temp. for 10 min. Then Ach. solution was added (0.110 g Ach. chlor 5 ml water) and the pH recorded every 5 m 20 min.

The molar concentration of an in reducing the activity of an enzyme by half is I_{50} and is a suitable constant to characteri inhibitor. By making use of Augustinsson's (Fig. 1) for competitive inhibition it is e determine the I_{50} value. This is done sim finding the concentration of Sch. correspond $V/V_1 = 2$. By this method Tammelin found the I_{50} for Sch. in serum to be 1,3 limol/L.

RESULTS

In Fig. 1 the inhibition of Ach. h lysis by serum as a function of Sch. co tration is plotted for 8 patients sh normal reactions to Sch. This is li The lines B and C represent the inhi of the serum of two patients showing longed apnea after Sch. injection.

In Table I the I_{50} values for the 8 pa are given as taken from the graph in F In Table II the I_{50} values for the pa



. Inhibition of serum cholinesterase as a
on of succinylcholine concentration. V =
on rate with uninhibited enzyme. V_1 =
on rate with inhibited enzyme. Line A:
from 8 patients reacting normally to suc-
oline injection. Lines B and C: Serum from
nts showing prolonged apnea after succinyl-
choline injection.

le I. *I_{50} for 8 patients reacting normally to Sch. injection.*

	Ach. hydrol. pH-change per 20 min.	I_{50} Millimol/L $V/V_1 = 2$
	0.22	1.45
	0.21	1.30
	0.19	1.40
	0.40	1.35
	0.15	1.33
	0.18	1.35
	0.20	1.33
	0.07	1.30
		Mean: 1.35

ng prolonged apnea are shown together
he approximate duration of the apnea.
ch. dose was approximately 100 mg.
the same method I found the I_{50} value
rse serum to be 1.75 millimoles/L, and

Table II. *I_{50} for 2 patients showing prolonged response to injected Sch.*

Apnea in min.	Ach. hydrol. pH-change per 20 min.	I_{50} Millimol/L $V/V_1 = 2$
30	0.19	2.00
180	0.07	30.00

for dog serum 2.65 millimoles/L. In this connection it is interesting to note that the duration of apnea in the dog is considerably longer than in the horse after the same dose Sch. per kg body weight (Thesleff, 1952).

DISCUSSION

It has been assumed that the esterase in serum hydrolysing Ach. was identical with the one hydrolysing Sch. Recent investigations by Foldes throw doubt on this. The present investigation shows that the affinity of the serum esterase for Sch. as expressed by the I_{50} value is not the same in the plasmas of horse, dog and man. In the plasmas of different humans it appears to be fairly constant, but two cases are reported where a marked increase in I_{50} values was found, and these cases showed prolonged apnea after Sch. injection.

SUMMARY

The inhibitory effect of Sch. on the serum cholinesterase of horse, dog and various human individuals was investigated by the electrometric method.

The inhibitory effect was found to be different for the three species. In 8 patients it was found fairly constant, but in 2 patients showing prolonged apnea after Sch. injec-

tions the inhibitory effect of Sch. was considerably reduced.

These findings support the theory of Foldes that there are either different enzymes responsible for Sch. and Ach. hydrolysis in serum, or that there are qualitative differences in this enzyme.

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THE URIC ACID CONCENTRATION IN SERUM ETERMINED BY ENZYMATIC SPECTROPHOTOMETRY

By S. GJØRUP, H. POULSEN and E. PRÆTORIUS

the Rigshospital, Med. Dept. A and the Department of Pharmacology, University of Copenhagen, Copenhagen, Denmark

(Received for publication March 6, 1955)

analytic results obtained by the hith- ıost commonly employed colorimetric trimetric reduction methods for uric etermination differ essentially, as de- rated by Lous & Sylvest (1954), from obtained by enzymatic measurements. ızymatic uric acid determination by olet spectrophotometry is becoming and more extensively used, being the exact method, it will be necessary to the uric acid concentrations that must arded as the limits of the normal range iation.

object of the present investigation has o procure an analytical material on the of which such a range of variation can ed, suitable for use in practice and e clinical diagnosis. It has been thought rational not to take account of the diet subjects examined or the time of awal of blood. Statistical calculations een included only as far as they were ed for the study.

MATERIAL AND METHODS

Material

investigations comprise 300 observations, ted on 143 male and 157 female subjects. e incidence appears from Table II. Part of the material has previously been published (Prætorius, 1951). The rest comprises healthy volunteers, nurses, medical students, and doctors, as well as patients admitted to the Medical Department A, the Rigshospital. Persons with a known familial predisposition to gouty arthritis and other diseases with a raised serum uric acid concentration, especially renal diseases, have been ruled out. Preliminary statistical calculations have shown Prætorius's original series and the supplementary series to be compatible.

As mentioned, no account was taken of the subject's diet or the time of withdrawal of blood. Each blood sample was analyzed by the authors on the day of withdrawal.

Analytic methods

The uric acid concentration was determined according to the enzymatic spectrophotometric method indicated by Prætorius & Poulsen (1953). A detailed account is given in this paper of the analytic technique employed. This has since been improved, however, by the following technical alterations, which have been found of practical value:

While previously the reference cell and the measuring cell contained different dilutions of serum, the contents of the two cells are now identical. Further, the optical density of the reference cell and its contents is defined by a read extinction of 0.200 (against previously 0.000), *i. e.* the galvanometer needle points at zero at the stated extinction when the reference cell is illuminated.

Extrapolation was performed at 3 to 5 extinction readings within one minute of addition of uricase.

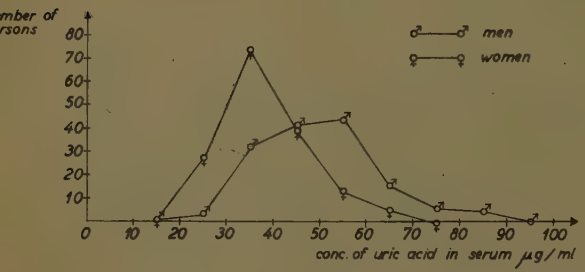

Fig. 1. The distribution of uric acid concentrations in sera from 143 men and 157 women. (Class range 10 µg/ml). Ordinates: number of subjects. Abscissae: serum uric acid concentration in µg/ml.

RESULTS

It appears from Table I that the serum uric acid concentration in men differs markedly from that in women, being highest in men, as is also the result of studies based on other methods.

It is a well-known fact that no limits can be fixed for the "normal" concentrations. If, however, we define the normal range of variation arbitrarily as the mean ± twice the *standard deviation of the single observation (s)*, then 97 per cent of the observations, as appears from Table III, fall within the range of 26—75 µg/ml for men and within the range of 20—57 µg/ml for women. Accordingly we should in clinical diagnosis be justified in regarding serum uric acid concentrations exceeding 75 µg/ml (= 7.5 mg per 100 ml) in men and 57 µg/ml (= 5.7 mg per 100 ml) in women as raised beyond the normal, since there is only 3 per cent chance that they are not.

No relationship was ascertained between the uric acid concentration in serum and age, either men or in women (Table II).

Fig. 1 illustrates the distribution of the observations. It is obvious that the concentration range is greater for men tha women. The curves plotted in Fig. 1 in the main the normal distribution c

Table I. *Serum uric acid concentra in men and women. Mean, standard d tion of the single observation (s), and s ard error of the mean (s. e. m.) expr in µg/ml.*

Sex	Number of obs.	Mean µg/ml	s µg/ml	s
Men	143	50.4	12.2	
Women	157	38.4	9.2	

Table II. *Age variations of the serum acid concentration expressed by mea µg/ml after division of the series 10-year-groups.*

	Men		Women	
Age years	Number of obs.	Serum uric acid µg/ml	Number of obs.	Se uri
20	3	54.0	6	
21— 30	36	50.2	20	
31— 40	19	51.4	24	
41— 50	13	51.9	17	
51— 60	16	50.4	16	
61— 70	27	48.1	23	
71— 80	21	52.1	37	
81— 90	8	47.9	12	
91—100	0		1	
100	0		1	

Table III. *Serum uric acid concent in men and women. Mean and normal in µg/ml. Number of observations the normal range of variation (mean ± 2 × s).*

Sex	Mean µg/ml	Normal range µg/ml	Numb obs. w normal
Women	38.4	20—57	138 =
Men	50.4	26—75	152 =

SUMMARY

ım samples from 143 men and 157
ı were examined for uric acid concen-
by enzymatic spectrophotometry. The
e concentrations were found to be 50.4
8.4 μg/ml, respectively. 97 per cent
observations fell within the range of
5 μg/ml for the men and 20—57 μg/ml
e women.

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THE ANTICOAGULANT ACTIVITY AND TOXICITY OF DEXTRAN SULPHATE AND A XYLAN POLYSULPHURIC ACID

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The toxicity of some synthetic heparin substitutes was recently studied (Astrup, 1953). We have now had the opportunity of investigating the toxicity and anticoagulant effect of a dextran sulphate of low molecular weight. Such a preparation was introduced as a nontoxic substitute for heparin (Walton, 1953, 1954). The results are presented here together with a study of a xylan polysulphuric acid ester (Thrombocid).

EXPERIMENTAL

Materials

Heparin standard: This was a sample of the provisional international standard heparin containing per definition 130 Toronto units per mg. It was supplied by the National Institute for Medical Research, London.

Heparin Leo: This was a commercial product kindly supplied by Messrs. Løvens kemiske Fabrik, Copenhagen.

Dextran sulphate: This compound was supplied by the National Institute for Medical Research, London. It had been prepared by Dr. C. R. Ricketts according to his description (1952) and had been proposed as a provisional standard for dextran sulphate. Further samples of a similar preparation (Batch 52-DS-039) were obtained from Dr. K. Walton, University of Birmingham. No difference was observed between these two samples.

Thrombocid: Samples of Thrombocid kindly placed at our disposal by Dr. Willi B München, Solln. This xylan sulphuric acid was included in our investigations because it to our notice that the xylan sulphuric acid which had been used in our previous investi (Astrup, 1953) was not identical with the compound marketed under the name *Thron* such as had been supposed.

The cellulose trisulphuric acid used in experiments was prepared as described prev (Astrup, Galsmar & Volkert, 1944).

Anticoagulant activity in vitro

The anticoagulant effects of the dif compounds were compared by means fowl plasma method described by Fisc Schmitz (1932). Fresh fowl plasma three times with Ringer solution was An extract of chick embryos, as ger used in tissue cultivation, was use thrombokinase. The 3rd or 4th extra the embryos with Tyrode solution sti a powerful thrombokinase effect and diluted about 1:50, so that 1 drop clot a mixture of 5 drops of chick p with 1 drop of Ringer solution in 50 seconds at 39°. The stock solutions kept in icewater during the experiment anticoagulant compound was dissolv Ringer solution in a concentration s

rop prolonged the clotting time of the a thrombokinase mixture to 5 to 7 es. From this stock solution appro- dilutions (in per cent of the stock on) were prepared with Ringer on. The results were recorded gra- ly with the concentration of the anti- lant solution (in per cent of the stock on) as abscissa and the logarithm of otting times (read as 1/100 minutes) dinate. In this graph heparin gives a ht line, and by comparison of the s of the curves obtained with different rations the relative potencies of the rations can be estimated. The slopes e curves depend on the properties of lasma and thrombokinase used, and a in preparation of known potency has fore to be used for comparison up & Jensen, 1938; Astrup, 1938).

comparison between the heparin ard and heparin Leo is presented in 1. The stock solutions used contained mg substance per 100 ml. The ard heparin gives in this experiment ope 2.59—1.95 (calculated for 100 per concentration as unit) = 0.64. Heparin gives the slope 2.65—1.95 = 0.61, and ins thus about $\frac{0.61 \times 100}{0.64} = 95$ per of the activity of the standard. The ard had 130 provisional international per mg, hence heparin Leo contained 123 units per mg according to this ation.

comparison between dextran sulphate eparin Leo is presented in Fig. 2. The solutions contained 5.0 mg heparin 7.9 mg dextran sulphate per 100 ml.

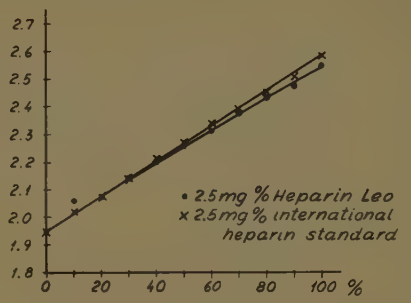


Fig. 1. Comparison between potency of heparin standard and of heparin Leo by the fowl plasma method.
Abscissa: Relative concentration of anticoagulant.
Ordinate: Logarithm of the clotting time.

It is apparent from the curves in this experiment that dextran sulphate does not produce the same inhibition curve as does heparin. The dextran sulphate curve deflects at higher concentrations. At smaller concentrations dextran sulphate has under these conditions about half the activity of the heparin sample.

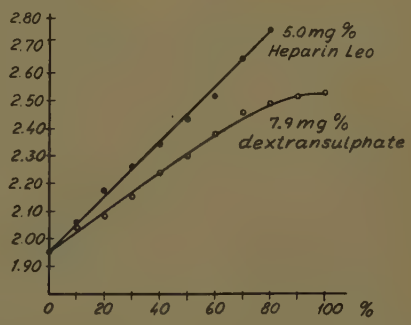


Fig. 2. Comparison between potency of heparin and of a dextran sulphate by the fowl plasma method. For description see Fig. 1.

A comparison between the effects of heparin and Thrombocid appears from Fig. 3. Stock solutions (per 100 ml):

Heparin 2.5 mg; Thrombocid 10.0 mg. The Thrombocid curve differs from the heparin curve qualitatively as well as quantitatively. In so far as a comparison in potency can be made, Thrombocid as about 1/3 of the potency of heparin estimated after this method.

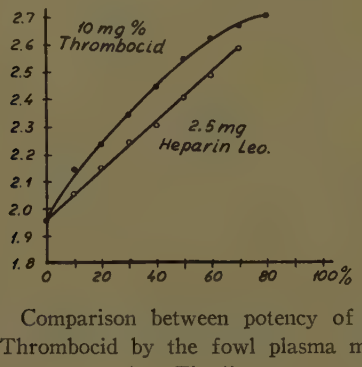


Fig. 3. Comparison between potency of heparin and Thrombocid by the fowl plasma method (see Fig. 1).

Anticoagulant effect in vivo

The anticoagulant effect *in vivo* was estimated as described by Piper (1946 a) (with some modifications). The rabbits were anesthetized with urethane (10 ml 20 per cent solution per kg orally) supplemented with ether if necessary. A silicone treated glass cannula was introduced in the carotid artery. The anticoagulant, dissolved in 2—5 ml Ringer, was injected through the other carotid. Blood samples were collected in silicone treated tubes. (The silicone treatment was made repeatedly with a 3 per cent silicone solution in petrolether and drying at 120°.) Clotting times were estimated in capillary tubes (30 cm long) as described by Piper *(l. c.)*. For each experiment a new animal was used. This procedure naturally introduces an individual variation, but excludes any influence on the results of a

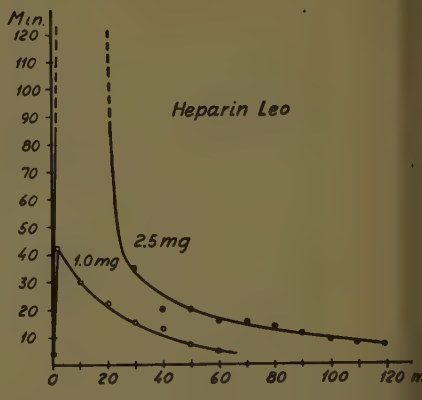


Fig. 4. Anticoagulant effect of heparin Leo *in* *Abscissa:* Time after injection. *Ordinate:* cl times in minutes. Doses applied: 1 mg and 2 per kg.

previous treatment. The curves pres are representative experiments.

Fig. 4 shows the effect of heparin L two doses (1 mg and 2.5 mg per kg ra These curves should be compared wit following curves obtained with de sulphate and Thrombocid.

The results of experiments with ferent amounts of dextran sulphate a from Fig. 5. Comparing the c obtained with those in Fig. 4 it is see they are not identical in shape. The d sulphate curves obtained here are similar to those published by W (1951). With due regard to the diff shapes, the anticoagulant activity esti *in vivo* by this method corresponds t effect observed *in vitro* by Ricketts (and Walton (1952) using the sheep p method of Kuizenga, Nelson & Ca (1943). They found the activity about 1/7 of that of heparin. This is not in accordance with the re

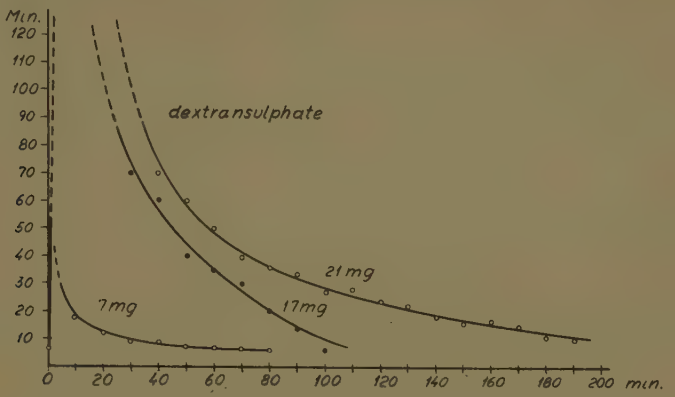
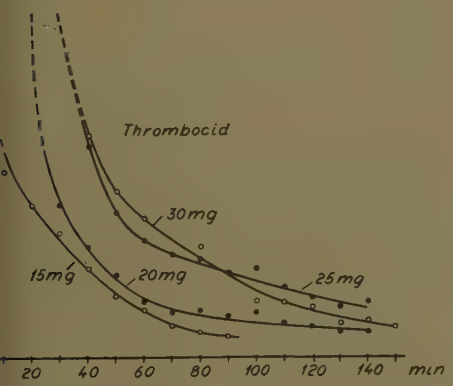


Fig. 5. Anticoagulant effect *in vivo* of dextran sulphate. Doses applied per kg: 7 mg, 17 mg and 21 mg.

ies as estimated in the above section the fowl plasma method, where in showed only about twice the y of dextran sulphate.



Anticoagulant effect *in vivo* of Throm- Doses applied per kg: 15 mg, 20 mg, 25 mg, 30 mg.

periments with Thrombocid are ted in Fig. 6. It is seen that Throm- is less active than dextran sulphate. disappearance of the anticoagulant y follows very much the same than in experiments with heparin or dextran were found to be larger for this compound than in experiments with heparin or dextran sulphate.

Effect on platelets in vitro

The platelet agglutinating effect *in vitro* was estimated on citrated rabbit blood obtained by heart puncture (compare Astrup, 1953). Solutions containing 0.1 and 5 per cent of the anticoagulant in 0.9 per cent NaCl were prepared. Rabbit blood (2.5 ml) was added to silicone treated tubes containing 0.5 ml of 3.5 per cent citrate solution and 0.5 ml of the anticoagulant. After gentle mixing the tubes were left for sedimentation for one hour. This procedure gave more consistent results than the centrifugation used before. The counts of single platelets were made on a drop of the supernatant plasma with the phase contrast microscope (400 ×) after the platelets had been given sufficient time to sediment in the counting chamber (0.05 mm deep). Results are given in thousands of free

Table I. *Effect of anticoagulant solutions (in two different concentrations) on the number of free platelets (in thousands per cubic mm) in rabbit plasma.*

Concentration	0.1 per cent	0.5 per cent
Control	(450—500)	—
Heparin Leo	450—500	450—500
Dextran sulphate	450—500	450—500
Thombocid	250—300	150—200
Cellulose sulphate	20— 50	10— 20

platelets per cubic mm of the final plasma mixture. As is usual in platelet counts the individual variations are large, but Table I presents typical results.

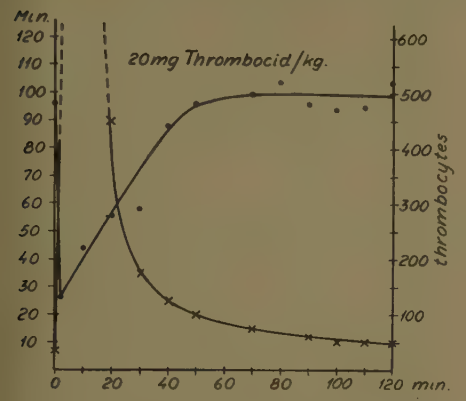
It is seen that neither heparin nor dextran sulphate produces platelet agglutination. Thrombocid produces a slight agglutination, which is very much lower than that produced by the cellulose sulphate used as control.

Table II. *Platelet agglutination on rabbit blood in vivo of he dextran sulphate and Thrombocid. Pla in thousands per cubic mm.*

Time in minutes for removal of sample	Heparin 1 mg/kg	Dextran sulphate 15 mg/kg	Thrombocid 20 mg/kg
0	450	650	400
2	500	680	380
10	487	580	440
20	472	620	410
30	460	680	490
40	439	560	410
50	455	600	500
60	—	520	460
70		530	420
80		510	—
90		592	
100		581	
110		597	
120		580	

Effects on platelets in vivo

These experiments were performed at the same time as the estimation of the anticoagulant effect *in vivo*. 2.5 ml blood were added to tubes (siliconized) containing 0.5 ml 3.5 per cent citrate solution. After sedimentation for one hour the platelets were counted as mentioned above. A slight clot formation or an ineffective silicone treatment give irreproducible results. Heparin (1 mg/kg; 2.5 mg/kg; 3 experiments) did not produce any platelet agglutination. (See Table II.) Dextran sulphate (5 experiments: 7, 15, 17, 21 and 30 mg per kg) did not produce any agglutination in a period of two hours (see Table II). The investigation of Thrombocid (8 experiments; Table II) presented difficulties. Two experi (15 and 20 mg/kg) produced no aggl tion. Three experiments (20, 25 a mg/kg) gave a very considerable aggl tion, so that no reappearance of the pl in the blood occurred in the 2 hours of the experiment. In three other e ments with Thrombocid (20, 25 and 3 kg) reappearance occurred such a described by Piper (1945, 1946 b) experiment with 20 mg/kg is pre in Fig. 7. A control experiment wit lulose sulphuric acid gave results corr ding with those of Piper. The experi with Thrombocid thus gave very incon results.



7. Disappearance and reappearance of throm-
es after application of 20 mg/kg Thrombocid
ved with time and estimation of the clotting
Abscissa: Time in minutes after injection.
nate: Left. Clotting time in minutes. *Right.*
ber of platelets in thousands per cubic mm.

DISCUSSION

ne results of the present investigation
that the sample of dextran sulphate
ied does not produce platelet agglutina-
It can be considered nontoxic to the
nism according to this test. These
ts confirm the conclusions of the British
stigators. The sample of dextran sul-
e here investigated is the first synthetic
oagulant of the heparin type, which in
hands has stood to the full extent the
let agglutination test for toxicity.
ently the molecular weight plays a large
in this effect. The British workers
d samples of dextran sulphate of higher
cular weight (40 000) to be toxic in
dance with the results of Grönwall,
lman & Mosimann (1945).

ne anticoagulant effect of dextran sul-
e is only about one seventh of the
t of heparin when estimated on rabbit
l *in vivo*. Compared by the fowl plasma method the activity was estimated to about one half of that of heparin. This result shows that the effects of heparin and dextran sulphate on the components of the blood clotting system are not identical, and that results obtained by different methods may not be comparable. This is in accordance with earlier comparisons between heparin and synthetic anticoagulants. Differences in the shape of curves of different anticoagulants estimated after the fowl plasma method were described by Astrup (1938) and Fischer & Astrup (1938) and confirmed by Piper (1946 a) in the case of sulphuric acid esters of cellulose, chitin and starch. Similar results were observed with ox plasma, Astrup & Galsmar (1944). It is interesting that dextran sulphate also shows a difference in action in this respect because it otherwise behaves much like heparin. Using the methods described by Blombäck, Blombäck, Corneliusson & Jorpes (1953) it was found by Yamashina (1954) that even different types of heparin (β-heparin) differed in their anticoagulant action. Though our results with rabbit blood *in vivo* compare with those obtained by the British workers using sheep blood *in vitro,* the results obtained with fowl blood show that heparin cannot be used as a standard of reference for comparison with dextran sulphate. As far as practical results are concerned, a comparison between the effects of heparin and dextran sulphate has to be performed on human patients. It appears that the lack of platelet agglutinating effect of dextran sulphate warrants its trial as an anticoagulant in the clinic, though it has to be considered that much larger doses of dextran sulphate have to be used than of heparin.

Such a trial has been performed by Ricketts, Walton, van Leuven, Birbeck, Brown, Kennedy & Burt (1953).

Concerning the sample of "Thrombocid", this compound is relatively nontoxic in studies of the platelet effect *in vitro*. It is less toxic than the xylan compound previously studied which produced considerable agglutination in rabbit blood *in vitro*. However, the results of the experiments *in vivo* show that Thrombocid can cause considerable agglutination of the platelets, though large individual variations in effect and toxicity were encountered. Husemann, Pfannemüller, , Hertlein & Hoffmann (1954) have recently studied the accumulation and excretion *in vivo* of heparin and xylan sulphuric acids of different polymerization degree and sulphuric acid content.

SUMMARY

The anticoagulant effects of dextran sulphate and Thrombocid cannot be measured in terms of the effect of heparin.

The activities of various anticoagulants depend upon the experimental conditions used. Qualitative comparable results are not always obtainable.

Dextran sulphate of low molecular weight shows no platelet agglutinating effect on rabbit blood *in vitro* or *in vivo*.

Thrombocid (a sample of xylan sulphuric acid) produces very little platelet agglutination in rabbit blood *in vitro*, but can produce considerable agglutination *in vivo*.

ACKNOWLEDGMENT

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THE INCORPORATION OF P^{32} IN PHOSPHOLIPID FRACTIONS IN MAN

(A PRELIMINARY REPORT)

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INTRODUCTION

The importance of the plasmalipids in the development of various vascular and endocrine diseases is generally acknowledged. The role of the phospholipids has been thought to be connected with the physicochemical stability of the lipoproteins of the blood, perhaps also of the lipids in the vascular walls (Ahrens & Kunkel, 1949).

"Phospholipid", is, however, a general term covering several chemical substances, some of which are still poorly defined. In these departments important differences have been found between the variations of lecithin, cephalin and sphingomyelin during various physiological states and in the course of various diseases (Posborg Petersen, 1952). It therefore seems of interest to go beyond the simple determination of total phospholipid and to try to obtain more information about the individual fractions.

Investigations of P^{32} incorporation in *total plasma phospholipid* in the human being have been carried out by Cornatzer & Cayer (1950) and Balfour (1947), who concluded that the adjusted specific activity was about the same at extremely different plasma phospholipid levels. This probably indicates that the turnover rate of phospholipid is proportional to the plasma level.

Investigations of P^{32} incorporation phospholipid *fractions* have only been formed in animal experiments. It has shown *e. g.* that the synthesis of the var fractions of phospholipids in the kidney liver decreases in the following order: thin, cephalin, sphingomyelin (Hevesy Hahn, 1940; Zilversmit, Entenman & C koff, 1948).

The following is a preliminary repor investigations of the incorporation of into two fractions of the plasma phos lipid in patients with various vasc diseases.

The purpose of this communicatio mainly to discuss the technical aspects of methods employed.

PROCEDURE

10 to 25 μC of labelled H_3PO_4 in 5 to 1 of physiologic saline (pH 7) was given intra ously to the patients. The phosphorus conte total phospolipid, phosphoglyceride and sph myelin, as well as the radioactivity of these tions were determined 7 to 8 times during course of 6 to 8 days, in some cases for a c of weeks.

One 50 ml sample of heparinized blood is for duplicate chemical analyses and duplicate vity determinations of the various fractions. mediately after withdrawal the plasma is sepa by centrifugation. 20 ml of plasma are tr

ml of 10 per cent trichloroacetic acid (TCA). ecipitate is filtered off and washed with 10 nt TCA, the washings being added to the to make up 200 ml.

Inorganic phosphorus. The TCA filtrate ded for the estimation of inorganic phos- and for the activity determination of in- phosphorus. 4 ml are used for the deter- on of inorganic phosphorus by a modified Subbarow method, using 2,4-diaminophenol- hloride (Amidol) as reducing agent.

l are used for the precipitation of inorganic orus with an ammoniacal magnesium citrate n (Fiske, 1921, Mathieson, 1909) adding phosphate to yield a precipitate of 100 mg. recipitate is collected on small perforated um dishes, covered with filter paper and d on a simple suction aggregate. The dishes cked dry with a few ml of absolute alcohol unted in an ordinary G. M. counter.

The TCA precipitate on the filter paper is ed in an Erlenmeyer flask with 350 ml ing alcohol acetone. After cooling, the re- g protein is filtered off and washed with addi- solvent until a final volume of 400 ml is d.

Total phospholipid phosphorus. 50 ml of trate are evaporated and ashed with 1 ml of trated sulfuric acid and small amounts of gen peroxide 30 per cent. After adding a few distilled water and boiling for a moment, the n is made up to 50 ml. 4 ml are used for the inations of phosphorus and 46 ml for the tation and activity determination as de- above.

Phosphoglyceride phosphorus and sphingo- phosphorus. 150 ml of the filtrate from B. porated to 4 ml and the lipids partially hydro- with 15 ml n KOH at 37° C for 16 hours idt, Benotti, Hershman & Thannhauser). Then f 5 n HCL and 18 ml of 10 per cent TCA are After 3 hours the mixture is filtered and the lipid precipitate is washed with additional 10 per cent TCA.

E. The filtrate, containing the phosphate obtained by the hydrolysis of phosphoglycerides, is evaporated to dryness, ashed with 2 ml of concentrated sulfuric acid and finally made up to 100 ml. Again 4 ml are used for the phosphorus determination and the remaining 96 ml for the precipitation and radioactivity measurement.

F. The precipitate from D., containing the non-hydrolyzed *sphingomyelin,* is extracted with 150 ml alcohol acetone in a Kjeldahl flask. The extract is transferred to another flask, evaporated to dryness and ashed as described above. After making up to 50 ml, the solution is again divided in 4 ml and 46 ml portions for the chemical and for the activity determinations.

COMMENTS ON PROCEDURE

Extraction. In a series of experiments it has been shown that there is very close agreement between the values for the lipid phosphorus content in the extract prepared from the trichloroacetic acid precipitate obtained from plasma and the lipid phosphorus content of alcohol acetone extract prepared directly from plasma.

Separation problems. To check the accuracy of the separation of the TCA precipitate from the activity of the filtrate, some experiments have been carried out in which a very high activity was added to normal plasma. After precipitation and washings as indicated above, practically no activity was found in the precipitate.

Furthermore, when labelled inorganic phosphate was added before the hydrolysis, it was found that practically nothing entered into sphingomyeline.

The accuracy of the total procedure was further checked by collecting the rinsing water from all glass material used in a complete run. After evaporation and precipitation counting for 24 hours gave values identical with the background.

If the activity determinations are precise, it should of course be possible to omit the determination of the total phospholipid activity as this value

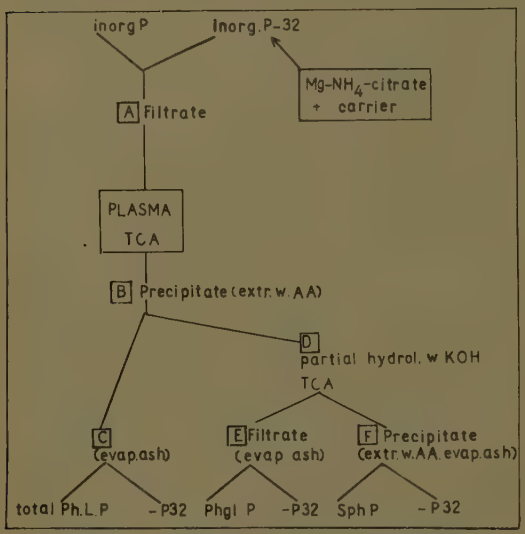


Fig. 1. Flow sheat of the procedure.

can be calculated from the absolute amounts of phosphorus and the activities of the two fractions. Fig. 2 shows a comparison of actual determinations of total phospholipid activity and the calculated activities. The good agreement gives further evidence of the reliability of the method.

Counting. The activity of the various fractions is determined on the same final substance (magnesium-ammonium-phosphate), which is spread homogeneously on identical backing material and counted in a fixed position with respect to the G-M-tube.

Fig. 2. Correlation between measured and calculated total phospholipid activity.

Before precipitation enough carrier phos is added to make up just about 100 mg on aluminum dish, the variations being so small than 5 mg/cm^2), that selfabsorption correctio be neglected. This precipitation-filtration dure yields activity estimation within ± 1 pe in the duplicates.

Calculations. The specific activities o phospholipid fractions have been expresse "adjusted specific activities". The incorpo of P^{32} into the plasma phospholipids is of *dependent* upon the activity of the plasma ino phosphorus — although probably not deter solely by it. As the activities of inorganic phorus obtained after injection of P^{32} in indi patients differ somewhat, it is reasonable to the phospholipid activities by means of organic phosphorus activities. Other investi (Balfour, 1947; Cornatzer & Cayer, 1950) chosen the 24 hours value of inorganic phosp activity for determination of the correction

We have preferred to use a correction obtained in the following way.

Six consecutive inorganic phosphorus act found in one arbitrarily selected individual No. 3) were chosen as test values. The rati tween these and the corresponding inorganic phorus activity in each of the other patients calculated.

The average value of these six ratios correction factor. The following figures typical example

2.26 — 2.34 — 2.49 — 2.46 — 2.39 — 2.33 : 2

In this way the specific activities of th fractions are expressed as if inorganic ac of all the patients had been the same as patient arbitrarily chosen as a test case.

RESULTS

In Fig. 3 are shown the results ob in a "normal" man aged 49 years. A rise in the phosphoglyceride phosp activity is seen, the peak being reach about 3 days. Then a rather steep fall

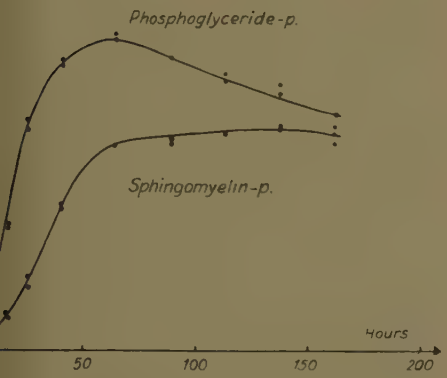
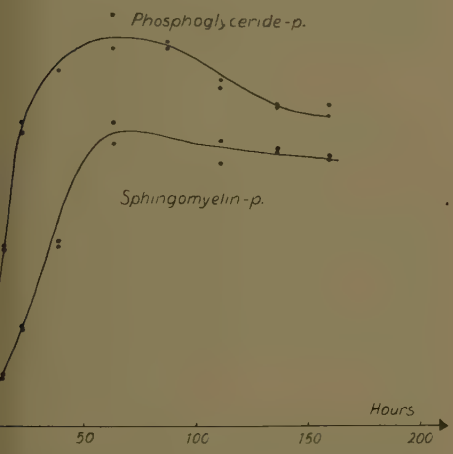


Fig. 3. Normal man, aged 49 years.



4. 57 year old man. Coronary occlusion weeks before. Intermittent claudication.

g the next 150 hours. The sphingo-
n phosphorus curve is seen to be lower:
se is less steep and there is something
plateau between 75 and 150 hours —
ps a slight fall at the end.
. 4 shows the incorporations in a 57
old man with atherosclerosis. He had
ute coronary occlusion 6 weeks before
ad claudications of the legs (5 years).

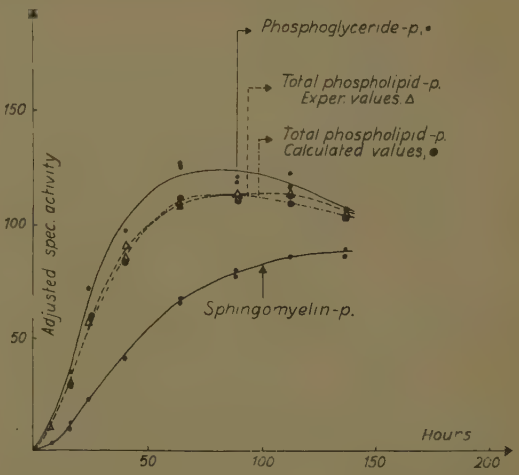


Fig. 5. 29 year old woman. Diabetes mellitus of 25 years duration. Proliferative retinopathy. Kimmelstiel Wilson's syndrome.

These curves seem to be somewhat higher, but the general form and the relationship between the two curves is similar to the normal one.

Fig. 5 is from a 29 year old woman with severe diabetic angiopathy and Kimmelstiel-Wilson syndrome.

The sphingomyelin curve rises throughout the 150-hour period. Here also the calculated and measured values of total phospholipid phosphorus activity is shown, giving good agreement.

Fig. 6 shows the result obtained in another long term diabetic patient with severe kidney lesions. The two full drawn curves are to be compared with the last figure. Also here the sphingomyelin curve rises continually. The stippled curves show the results after estrogen administration. No distinct peak value is obtained during the experiment, either on the phosphoglyceride or on the sphingomyelin curve.

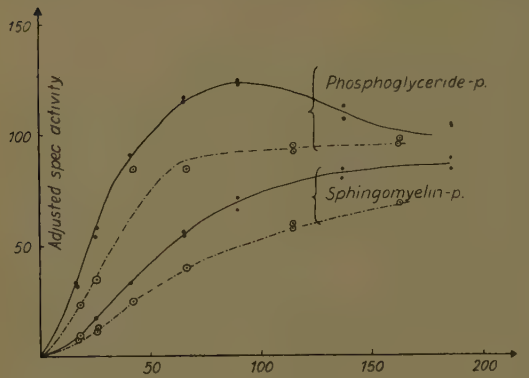


Fig. 6. 65 year old woman. Diabetes mellitus of 15 years duration. Diabetic retinopathy. Kimmelstiel Wilson's syndrome. Stippled curve: 14 days after the administration of 2 mg of stilbestrol daily.

Fig. 7 shows the results obtained in a patient with essential hyperlipemia on two different diets. Both curves are less steep and lower on the low fat diet.

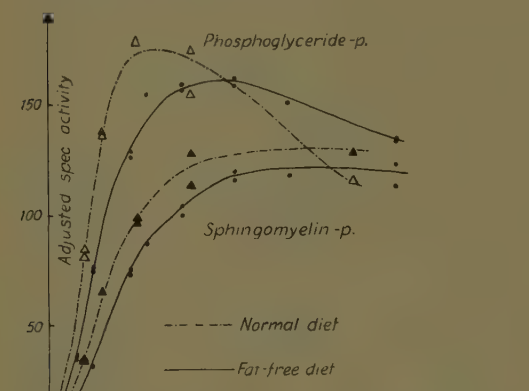


Fig. 7. 56 year old woman. Essential hyperlipemia.

DISCUSSION

The methods employed in these experiments allow us to make an accurate determination of the incorporation curve of P^{32} in two phospholipid fractions of the plasma, after the administration of only quite small d of activity, *i. e.* 10—20 μC.

The most striking result hitherto obta has been the constant difference betwee incorporation curve of phosphoglyceride sphingomyelin. It seems reasonable assume that sphingomyelin is synthes at a much slower rate than is phosphog ride. This fact has not been demonstr previously in the human being, but it accordance with results from animal ex ments (Hevesy & Hahn, 1940; Zilver Entenman & Chaikoff, 1948).

One of the purposes of our investiga is to examine whether the synthesis of phospholipid fractions — as it is expre by the P^{32} incorporation curve — is diff in various diseases and during va physiological states.

In the preliminary studies reported there seems to be some difference bet the curves obtained from patients various diseases and in various cli states. Further investigations along lines are in progress in this laborator

SUMMARY

A method is described by which th corporation of labelled phosphate into fractions of plasma phospholipid (pho glyceride and sphingomyelin) can be foll with considerable accuracy after the ad stration of even quite small doses of P

Preliminary results obtained with method seem to indicate a difference i metabolism of these two fractions in h beings, the increase in the specific ac of sphingomyelin being markedly delay comparison with that of the phospho rides.

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ANTIHEMOPHILIC ACTIVITY IN COHN'S FRACTION PREPARED FROM POSTPARTUM SERUM

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The most probable cause of the abnormal bleeding tendency in congenital hemophilia is a genetically determined inability of the organism to synthetize a certain factor of a globulin nature. This so-called antihemophilic globulin (AHG) is necessary for a normal formation of thromboplastin in the blood. The defect in the coagulation mechanism in hemophiliacs can be corrected temporarily by the transfusion of fresh normal blood, fresh, frozen (— 25° C) or dried plasma prepared from non-hemophilic blood within 3 hours of removal, or by intravenous or intramuscular injection of certain fractions of plasma containing AHG in concentrated form, usually Cohn's fraction I. The ultimate goal of the treatment of hemophilia is to maintain the AHG titre of the blood on a sufficiently high level to prevent both spontaneous bleeding and an abnormal tendency to hemorrhage after trauma. Such substitution therapy would require daily administration of such a large quantity of AHG that it would be impracticable with current techniques to produce sufficient amounts from human plasma. It is, however, possible to use bovine AHG (Macfarlane, Biggs & Bidwell, 1954), particularly since it is considerably more active and stable than human AHG (Spaet & Kinsell, 1953). Nevertheless if bovine AHG can be produced in highly purified form than hitherto, its genic activity is presumably so high t use, other than temporarily, would too great a risk. Thus, it would be a advantage if AHG-like activity co derived from human postpartum bloo experience indicates that this is possi

One of us (K.-M. H.) found in *in* tests in 1952—1953 that small quanti Cohn's fraction I (Fr. I) prepared postpartum serum (PPS) were able t malize the coagulation time in hem blood. This observation was confirm experiments made in 1954, when the was also tested of Fr. I prepared from plasma (NP) and normal serum (NS blood donors. The fractionation of PP and NS was performed by A/B Kabi, holm, according to Cohn's method 6 Strong, Hughes, Mulford, Ashworth, & Taylor, 1946) for the fractionat plasma protein. For the past few gamma globulin and albumin hav among the preparations produced b Kabi with this method, and investig are now in progress with a view to pro AHG for therapeutic use from PPS.

MATERIAL AND METHODS

artum serum (PPS) was obtained from um blood (PPB) by centrifugation (2000 or 45 min.) at 4° C. This PPB contains, on to the blood lost during and after expul- the placenta, the blood from the cord after been severed. PPB is collected routinely, sterile conditions as possible, at the obste- artments of the Stockholm hospitals. Only om healthy women and uncomplicated deli- kept. The actual PPB is, on an average, ml per delivery, whereas the cord blood , as a rule, to only 10—20 ml. This is be- ne cord is not usually severed until after sations have ceased (Malmnäs & Nihlén, The blood is kept in a refrigerator (at in special 500 ml bottles containing peni- d dihydrostreptomycin in dry form. The delivered three times a week to A/B Kabi, it is centrifuged at once; the serum is zen and stored at —25° C.

al plasma (NP) and *normal serum* (NS) tained from the blood of 5 donors (4 non- women and 1 man, all between 20 and old). In no case was the coagulation time d when the blood was drawn. None of ects had ever exhibited an abnormal ten- o hemorrhage, nor had any of them a blood transfusion on any occasion. The as drawn from all five donors within 1½ 00 ml in each case; 200 ml were collected tle containing ACD solution and 200 ml in y bottle. The blood was chilled immediately ater; it was kept at this temperature and ntrifuged (2000 r.p.m. for 45 min.) at hin 3 hours of withdrawal. As soon as the and serum had been siphoned off with the vacuum under aseptic conditions, fractiona- started. Thus, withdrawal, centrifugation tionation were performed consecutively on e day (Nov. 29, 1954). From the 1000 ml collected in ACD solution, 620 ml of were obtained; the total protein content was 60.0 g per liter). The amount recovered was 2.15 g (5.8 per cent). From the g 1000 ml of blood, 480 ml of serum were ; the total protein amounted to 37.7 g (78.6 g per liter), of which 0.54 g (1.4 per cent) was recovered in Fr. I. As a comparison, it may be mentioned that 500 ml of PPS, fractionated simultaneously, contained 33.0 g of total protein, 1.4 g (4.2 per cent) being recovered in Fr. I. — The amount of protein was determined by analysis of the nitrogen content with Kjeldahl's method.

The different batches of Fr. I were supplied to us as the freeze-dried substance. This was dissolved in 0.9 per cent NaCl solution, and two dilutions with a 0.5 and 0.005 per cent protein content, respectively, were tested. Between the experiments, they were kept in a refrigerator at 2—4° C.

Hemophilic blood was taken from 5 boys (C. S., S.M., R.K., G.S., K.Å.); their age ranged from 3 to 11 years. They all presented the clinical features typical of hemophilia, and had a prolonged coagulation time in whole blood (CT) and in plasma (PCT). The platelet count, bleeding time, clot retraction time, prothrombin time, fibrinogen content and capillary resistance tested according to Göthlin had all been normal on several occasions during the preceding years. The CT in mixtures of normal and hemophilic blood (80, 50 and 20 per cent) was in every case the same as in normal blood alone (December 1954). The PCT did not become normalized when half the quantity of plasma from one hemophiliac was replaced by plasma from one of the others, but it was normal in a mixture consisting of equal parts of hemophilic and normal plasma. Consequently, it is not probable that the hemorrhagic diathesis was due in any of the cases to the presence of circulating anticoagulants, or to a PTC deficiency.

The coagulation time (CT) was determined on broad lines according to Howell, Lee & White (Behrendt, 1949). The blood was withdrawn from the antecubital vein, with light application of a tourniquet, using a dry, sterile glass syringe (10 or 20 ml) and a No. 2 needle. The blood was used only if the vein could be entered at once, and the quantity of air in the syringe was minimal. As soon as sufficient blood had been aspirated, it was transferred carefully from the syringe into well rinsed and completely dry glass tubes (8.0—8.5 × 100 mm), previously washed with a solution of potassium dichromate in sulphuric acid. The tubes, each

containing 1 ml of blood, were placed immediately in a water bath at 37.5° C. Each tube was tilted gently from the vertical to the horizontal position once every ½ minute for 10 minutes, and thereafter once a minute. The CT was estimated as the interval between the time the blood first entered the syringe and the end-point of coagulation, *i. e.*, when blood no longer flowed down the tube when it was tilted. Using this method, the CT ranged from 3.5 to 6.5 minutes in 20 children (4—15 years old) with no abnormal bleeding tendency. Duplicate determinations were made in all these controls; in 9 cases the CT was the same in both tubes, in 9 it differed by ½ minute and in 2 by 1 minute.

The effect of the different batches of Fr. I on the CT of hemophilic blood was tested in 0.1 ml of the respective solutions (0.5 and 0.005 per cent). This quantity was transferred to the required number of tubes immediately before the blood (1 ml) was added.

The plasma coagulation time (PCT) was determined in plasma prepared from 5 ml of oxalated blood (venous blood drawn into a 10 ml bottle containing a 1 × 5 cm piece of gauze impregnated with lithium oxalate). Immediately after drawing the blood, the bottles were placed in the refrigerator at 2—4° C; centrifugation (3000—3500 r.p.m. for 10 min.) was performed within one hour. The plasma was carefully pipetted off, leaving a layer about 5 mm thick. In well washed centrifuge tubes (15 × 95 mm), 0.1 ml of plasma was mixed with 0.1 ml of 0.9 per cent NaCl solution. The tubes were immediately placed in a water bath at 37.5° C, and 0.1 ml of $CaCl_2$ in 0.334 per cent solution was added. The tubes were tilted once every ½ minute. The PCT was estimated as the time elapsing between addition of the calcium solution and complete gelation of the plasma mixture. In the aforementioned 20 controls, the PCT determined in this way ranged from 1.5 to 3.5 minutes. Duplicate determinations were made in every case; the PCT showed the same value in both tubes, or differed by at most ½ minute.

The effect of the different batches of Fr. I on the PCT of 3 of the hemophiliacs was studied by substituting 0.1 ml of the respective dilutions (0.5 and 0.005 per cent) for the NaCl solution (0.1 ml).

RESULTS

Tables I—III show the effect on t of hemophilic blood of three different b of Fr. I prepared from PPS on Feb. 14 (Kabi 273),[1] Dec. 1, 1952 (Kabi 44 June 15, 1953 (Kabi 501). The fi batches (see Tables I and II) were

Table I. *Effect on the coagulatio of hemophilic blood of Cohn's fra (Fr. I) prepared from postpartum (PPS) on Feb. 14, 1952. In this a following tables, — denotes that no te made, and xx that the sample was dis*

Patient	Date	Coagulation time (min 1 ml of hemophilic bl		
		Without addition	0.1 ml of 0.5% Fr.I (PPS) added	0.1 0.00 (a
K.Å.	28.3.-52	39.0 40.0	5.0 6.0	16
G.S.	11.3.-52	32.0 —	4.5 4.5	13
G.S.	8.5.-52	21.0 30.0	4.0 4.5	14
R.K.	21.2.-52	49.0 —	4.0 4.0	30
S.M.	20.2.-52	28.0 —	3.0 —	—

Table II. *Effect on the coagulatio of hemophilic blood of Cohn's fra (Fr. I) prepared from postpartum (PPS) on Dec. 1, 1952.*

Patient	Date	Coagulation time (mi 1 ml of hemophilic b		
		Without addition	0.1 ml of 0.5% Fr.I (PPS) added	0.5 0.0 (a
K.Å.	15.4.-53	48.0 52.0	4.0 5.0	11
G.S.	17.4.-53	18.0 20.0	4.0 5.0	7
R.K.	10.4.-53	55.0 —	7.0 7.5	15

[1] These and the subsequent figures in denote the number of the relevant batch Kabi's laboratories.

eze-dried substance at 0° C until they dissolved in physiologic saline; this one on the same day the first test with elevant preparation was made. The tested in January 1954 (see Table III) ored as freeze-dried substance at 0° C ½ months. It was then kept at room rature for 4½ months, until it was dissolved in physiologic saline on the day of the first test (Jan. 22, 1954).

The comparative effect on the CT of hemophilic blood produced by Fr. I prepared from PPS (Kabi 647), NP (Kabi 648) and NS (Kabi 649) fractionated on Nov. 29, 1954 is recorded in Table IV. These batches of Fr. I, as well as two others prepared from PPS on Sept. 28, 1953 (Kabi 516) and May 10, 1954 (Kabi 646), which were tested at the same time, had been stored as freeze-dried substance at 0° C until the day the tests were started, when they were dissolved in physiologic saline. The decrease in the CT produced by the two last-mentioned batches was as good as that produced by the Fr. I from PPS (Kabi 647) recorded in the Table.

Table V shows the effect on the PCT of one of the hemophiliacs produced by Fr. I prepared from PPS, NP and NS on Nov. 29, 1954. It thus appears as if Fr. I produced from NS also contains some antihemophilic activity.

e III. *Effect on the coagulation time mophilic blood of Cohn's fraction I) prepared from postpartum serum (PPS) on June 15, 1953.*

t	Date	Coagulation time (min.) in 1 ml of hemophilic blood		
		Without addition	0.1 ml of 0.5% Fr.I (PPS) added	0.1 ml of 0.005% Fr.I (PPS) added
	27.1.-54	26.0 27.0	7.0 7.5	19.0 20.0
	30.1.-54	27.0 28.0	12.0 13.0	19.0 19.0
	22.1.-54	30.0 30.0	xx xx	17.0 17.0
	29.1.-54	90.0 —	14.0 —	25.0 —
	25.1.-54	25.0 26.0	10.0 11.0	18.0 20.0

ble IV. *Comparative effect on the coagulation time of hemophilic blood pro- d by Cohn's fraction I (Fr. I) prepared from postpartum serum (PPS), normal ma (NP) and normal serum (NS) on Nov. 29, 1954. In Case S.M., we can find xplanation of the increased coagulation time after additions of both Fr. I solutions from NS and after addition of 0.9 per cent NaCl.*

Date	Coagulation time (min.) in 1 ml of hemophilic blood							
	Without addition	0.1 ml of 0.5% Fr.I (PPS) added	0.1 ml of 0.005% Fr.I (PPS) added	0.1 ml of 0.5% Fr.I (NP) added	0.1 ml of 0.005% Fr.I (NP) added	0.1 ml of 0.5 % Fr.I (NS) added	0.1 ml of 0.005% Fr.I (NS) added	0.1 ml of 0.9% NaCl added
7.12.54	28.0 30.0	4.0 4.5	22.0 23.0	5.0 6.5	17.0 18.0	21.0 24.0	29.0 xx	26.0 28.0
8.12.54	43.0 xx	4.5 5.0	23.0 25.0	6.5 7.5	21.0 24.0	44.0 47.0	38.0 43.0	43.0 46.0
7.12.54	34.0 xx	7.0 7.5	22.0 27.0	7.5 8.5	17.0 18.0	33.0 36.0	31.0 32.0	33.0 35.0
9.12.54	32.0 36.0	8.0 8.5	23.0 24.0	10.0 10.0	31.0 32.0	35.0 40.0	36.0 40.0	38.0 42.0
8.12.54	29.0 30.0	5.5 5.5	21.0 21.0	7.0 8.5	16.0 17.0	61.0 66.0	66.0 72.0	54.0 xx
9.12.54	42.0 —	5.5 —	23.0 —	6.0 —	24.0 —	42.0 —	48.0 —	xx —

Table V. *Comparative effect on the coagulation time of hemophilic plasma prod by Cohn's fraction I (Fr. I) prepared from postpartum serum (PPS), normal pla (NP) and normal serum (NS) on Nov. 29, 1954.*

Patient	Date	Coagulation time (min.) after recalcification of 0.1 ml of hemophilic plasm						
		0.1 ml of 0.9% NaCl added	0.1 ml of 0.5% Fr.I (PPS)added	0.1 ml of 0.005% Fr.I (PPS)added	0.1 ml of 0.5% Fr.I (NP) added	0.1 ml of 0.005% Fr.I (NP) added	0.1 ml of 0.5% Fr.I (NS) added	0.1 0.005 (NS)
K.Å.	18.12.-54	15.0 16.0	2.0 2.0	4.0 5.0	2.5 2.5	9.0 9.0	2.5 3.0	16.0

PPB is not free from bacteria, but contains *B. coli* among other micro-organisms. Moreover, pyrogenic substances seem to be accumulated in Fr. I. Consequently, great difficulties were encountered in obtaining a pyrogen-free preparation for *in vivo* experiments. This is why only one such experiment has hitherto been made (Fig. 1). One of the hemophiliacs (R. K.) was, in the course of 9 days, given a total of 800 mg of protein from

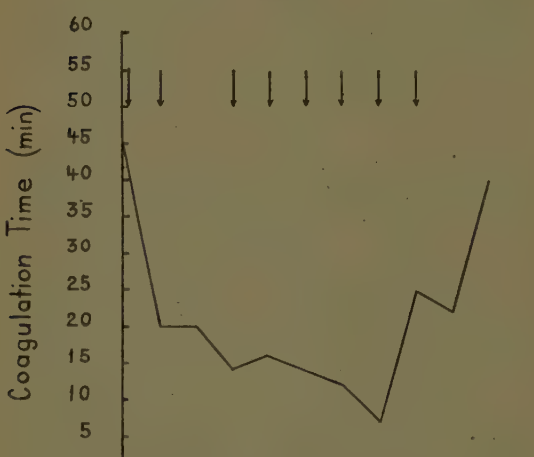

Fig. 1. Case of hemophilia (R. K., a 6-year-old boy, weighing 30 kg), treated with intramuscular injections of Cohn's fraction I prepared from postparum serum.
(↓ = intramuscular injection of 100 mg of protein).

a batch of Fr. I prepared from PPS on 27, 1952 (Kabi 296). It was stored at until the day of the first injection (April when it was dissolved in physiologic s He was given 8 intramuscular injec each consisting of 2 ml of solution conta 100 mg of protein. The CT (determin the capillary blood) decreased during ment, until the 8th day. The exper was, however, discontinued on the 9th since fever (38—38.5° C) developed w any definite explanation. The CT had on this day to slightly more than 20 mi from 7 minutes on the previous day.

Thus, it may be inferred from the r of these experiments that the antihemo activity of Fr. I prepared from PPS certainly as good as that of Fr. I from and considerably greater than that of from NS. Furthermore, Fr. I derived PPS is strikingly stable when stored as the dry substance, or in soluti 2—4° C.

DISCUSSION

It is an established fact that normal has only an inappreciable antihemo activity or none at all. The reason is sidered to be that the antihemophilic is consumed during coagulation of the It is therefore surprising that Cohn's fr

pared from the serum of postpartum is able to normalize the coagulation f hemophilic blood. A possible explana- s that, when delivery takes place, the content of the blood is so high that l of it is used up on coagulation, but remains in the serum. According to cola (1953), the activity of factor VII reased in the blood of women in the month of pregnancy. This may pos- apply to other factors necessary for lation as well. It must, however, be d out that Bendien & van Creveld) found an antihemophilic activity in ts from the placenta. Consequently, in sts, the antihemophilic activity of the rations of Cohn's fraction I derived postpartum serum might have been due e substance released from the placenta. rsely, the antihemophilic activity of the tal extract tested by Bendien & van ld might have derived from the post- n blood. It is our intention to make er study of the relevant problems, with ular reference to the degree of anti- hilic activity in the plasma, serum and rtum blood of newly delivered women. as not yet been possible to identify the mophilic factor, nor to prepare it in d form. The plasma derivate with the st antihemophilic potency is Cohn's n I. It nevertheless contains only 20 per cent of the antihemophilic y of the plasma, since the rest is lost actionation (Spaet & Kinsell, 1953). ntihemophilic factor is, however, more in this fraction than in plasma. Accord- Minot, Davidson, Lewis, Tagnon & r (1945), the coagulation time is d to an almost normal level in an adult hemophiliac within 30 minutes of the intravenous or intramuscular injection of 200—600 mg of protein from Cohn's fraction I. This effect was found to persist for 6 to 12 hours. Presumably, a considerably larger quantity is required to bring about a normal hemostatic mechanism (Macfarlane, Biggs & Bidwell, 1954). According to these authors, this probably necessitates administration of the amount of AHG present in about 2.5 liters of blood, and this dose must be repeated daily if the effect is to be maintained.

Cohn's fraction I prepared from plasma contains high-molecular proteins (60 per cent of fibrinogen) and should not, therefore, be administered in a higher concentration than about 5 per cent. Consequently, such continuous therapy is impracticable, partly on the grounds of the large volume that it would be necessary to inject. At present, it is not possible to produce from postpartum serum any appreciably higher concentration of AHG-like activity than that in the form of Cohn's fraction I. Our observation that postpartum serum possesses an antihemophilic activity is nevertheless not of theoretical interest alone. For, such basic material is both cheaper and more easily available than plasma from blood donors. Consequently, it would be a great advantage if postpartum serum could be used for the production of a substance with such activity.

SUMMARY

A normalization of the coagulation time of 1 ml of hemophilic blood and of 0.1 ml of hemophilic plasma was found to result from the addition of 0.1 ml of a 0.5 per cent protein solution from Cohn's fraction I, pre-

pared from the serum of postpartum blood, *i. e.*, the blood lost during and after expulsion of the placenta. Some reduction in the coagulation time was also obtained when the hemophilic blood and plasma were mixed with a 0.005 per cent solution.

The effect was possibly slightly more pronounced than that of the same quantity of protein from Cohn's fraction I prepared from normal plasma, and considerably greater than that of this fraction derived from normal serum.

It was confirmed by an *in vivo* experiment that Cohn's fraction I prepared from postpartum serum has an antihemophilic activity. Among the other findings were a higher total protein content (4.2 per cent) in fraction I prepared from postpartum serum in this fraction produced from normal s (1.4 per cent).

The antihemophilic activity of Cohn's tion I derived from postpartum serum found to be stable, both when stored a dry substance and in solution.

The theoretical and practical implic of these observations is briefly discuss

ACKNOWLEDGMENTS

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THE PROTEOLYTIC ACTIVITY OF GASTRIC JUICE

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kemura (1909) and Hirayama (1910) the first to show that certain pepsin arations had a marked proteolytic acti- in the weakly acid range. Later Will- er and Bamann (1929) observed the phenomenon with extracts of the gastric us membrane and considered that this eptic" proteolysis was due to the con- of leukocytes of the mucous membrane; atheptic activity was found in gastric

ace 1940 Freudenberg (1940) and s (1940) have reinvestigated the proper- f the protease of the stomach. Both of authors assume that the proteolytic ty in the weakly acid region is of a ptic rather than of a peptic nature. ever, they do not postulate the existence o different enzymes, but consider that eptic and catheptic functions are asso- l with different sites in the same mole- This view is suggested by the fact that e pepsin preparations studied — even rystalline ones — have catheptic activi- nat even surpass their peptic activities. e hypothesis is based upon the following vations:

Freudenberg and Buchs found a pH ivity curve with one peak at pH 2.2 more pronounced one at pH 3.3 (see Fig. 1). In most of their investigations the substrate was edestin. The extent of proteolysis was determined by nephelometric measurement of the turbidity caused by addition of sulfosalicylic acid to the reaction mixture after digestion. In their early work the same authors had found a pronounced fall in proteolytic activity at pH 2.7, but more recently Buchs (1953) and Tolckmitt (1954) have pointed out that this might be explained by the procedure used for adjusting the pH of the reaction mixture (pH values 3.33—2.73 and 2.2 were obtained by dissolution of the protein in n/7 CH_3COOH, 1 n CH_3COOH and n/50 HCl respectively, yielding solutions of widely differing ionic strengths). However in later studies where the edestin was dissolved in glycine -HCl buffers, Buchs still found two-peak activity curves with most rapid hydrolysis at pH 3 to 4.

Similar results were obtained by Demole & Milhaud (1951) and by Merten & Ratzer (1949). On the other hand, Geilenkirchen & Elbers (1950), Crebolder, Engel & Otten (1951) and Tolckmitt (1954) found a one-peak pH *vs.* activity curve.

2) As a further support of his view concerning a special catheptic function of pepsin, Buchs (1947) states that the catheptic acti-

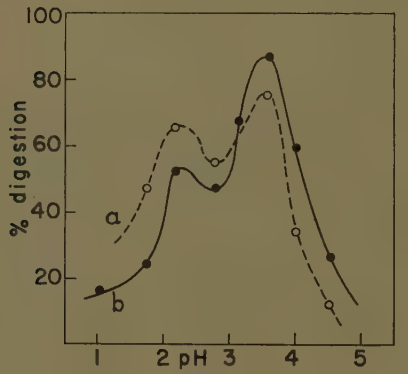

Fig. 1. (After Buchs.)
Curve a. Splitting of edestin at 40° C.
Curve b. Splitting of edestin at 63° C.

vity increases with increase in temperature whereas the peptic activity decreases (see Fig. 1). However, this may be explained in the following way: Below pH 3 denaturation of pepsin is so rapid and its rate increases so strongly with increasing temperature that the net result is a decrease in rate of hydrolysis. Above pH 3 this denaturation is less important so that the usual increase of rate of hydrolysis with temperature predominates. This has been stressed and supported by experimental evidence by Geilenkirchen & Elbers (1950) and by Crebolder *et al.* (1951), and Buchs's recent findings (1953) do not invalidate these objections.

3) Originally Buchs (1947) found that HCN and H_2S activated proteolysis far more strongly in the weakly acid than in the strongly acid region. He considered this further proof of the presence of a catheptic function.

In more recent experiments Buchs (1953) finds that the activators mentioned are effective over a very wide pH range. Furthermore the activation is relatively weak a obtained only at rather high concentrat of the activating substances. All this i it more probable that the effect observe in fact due to the influence of the activa upon the physico-chemical state of the strate, an interpretation which lends no port to the concept of a catheptic acti

Thus the main argument in favo Freudenberg and Buchs's concept of a ga cathepsin is their finding of a two-peak *vs.* activity curve with most pronounced vity at pH 3 to 4. However, as menti above there is no general agreement on point.

Merten, Schramm, Grassmann & Ha (1952) have endeavored to throw ligh this problem by some investigations in w they make use of preparative electropho on filter paper. They succeeded in rating, by electrophoresis of an extra gastric mucosa, a fraction having cath activity, a finding that is not in accord Buch's theory. Moreover, this cathepsin have been derived from leukocytes pr in the mucosa. Hence their findings d afford conclusive proof of the presen cathepsin in gastric juice.

Recently Heinrich (1953) carried paper electrophoretic studies of pepsin his results were at variance with tho Merten *et al.* in that he was not ab separate any cathepsin fraction.

Finally it must be borne in mind th their histochemical investigations of the teolytic enzyme of the gastric mucosa, H & Linderstrøm-Lang (1934) always f the rate of digestion of edestin to b lower at pH 3.3 than at pH 2.1.

will be seen from the foregoing that the ence of a catheptic activity in gastric — associated with or separate from the n — cannot be regarded as an estab- d fact. Since the problem is still being ssed, the author feels justified in pub- g some experiments intended to shed on this problem.

e description of the experiments will be ded by some remarks on determination oteolytic activity in general, which may interest in this connection.

several of the studies mentioned lenkirchen & Elbers, 1950; Tolckmitt,) proteolysis has been followed by elometric determination of the decrease nount of substrate, the undigested pro- being precipitated by addition of sulfo- ylic acid in the presence of gum arabic. ould be emphasized, however, that most ein precipitants precipitate not only un- olyzed molecules but also high molecular kdown products. For example, trichloro- c acid is able to precipitate protein frag- s with molecular weights as low as 5000 ler, Dodds, Phillips & Stephen, 1948). sequently the use of a nephelometric od for comparison of the rates of hydro- at different pH values must presuppose the high molecular hydrolysis products ed at the pH values in question all have ame tendency towards aggregation. If, ever, this tendency increases as the iso- ric point is approached, there is a possi- that the precipitate will become coarser, ucing a false impression of more exten- breakdown.

nsiderations of this kind leave doubt erning the suitability of a nephelometric method for elucidation of theoretical problems such as the establishment of a pH *vs.* activity curve.

Similar objections may be raised against other methods involving protein precipitation (determination of nonprotein nitrogen or of aromatic amino acids in the filtrate). It cannot be taken for granted that the substrate molecule is hydrolyzed the same way at different pH values. At one pH value medium sized polypeptide fragments may predominate; they will not be precipitated by trichloroacetic acid. At another pH value a mixture of small and large fragments may be formed; the large ones will be precipitated. In the latter case proteolysis will appear less extensive than in the former even though the number of peptide bonds hydrolyzed may have been greater in the former.

When fully denatured protein substrates are employed there is, however, fair agreement between the pH *vs.* activity curves found by precipitation procedures and those found by determination of the number of peptide bonds split (Korsgaard Christensen, 1952, 1955). This is probably due to the fact that, after all, the quantities of precipitate are determined accurately enough, whereas nephelometry in itself cannot be trusted to give reliable measures of the quantities of precipitate.

The above considerations lead to the conclusion that the best way of studying the problem of possible gastric cathepsin is to investigate the hydrolysis of various substrates by means of several different methods. Some of the experimental results of the author will be discussed in the following.

A. Do pure pepsin preparations possess a catheptic activity?

Certain earlier experiments afford a partial answer to this question. As for details, reference is made to an earlier publication (Korsgaard Christensen, 1955).

Fig. 2 illustrates the hydrolysis of native, of heat denatured, and of urea denatured egg albumin by a crystalline pepsin preparation (Armour). The native egg albumin is hydrolyzed most readily at a strongly acid reaction; at pH values exceeding 2 or 2.5 proteolysis is very slow. In contrast, the denatured preparations are hydrolyzed over a wide pH range. Urea denatured egg albumin is broken down with greater ease than heat denatured, probably because it is more profoundly denatured *i. e.*, with a more pronounced uncoiling of the peptide chains. Furthermore, curve c extends farther towards neutrality than does curve b.

This demonstrates the important circumstance that peptic hydrolysis at a weakly acid reaction is largely conditioned by the structure of the substrate molecule. The fact

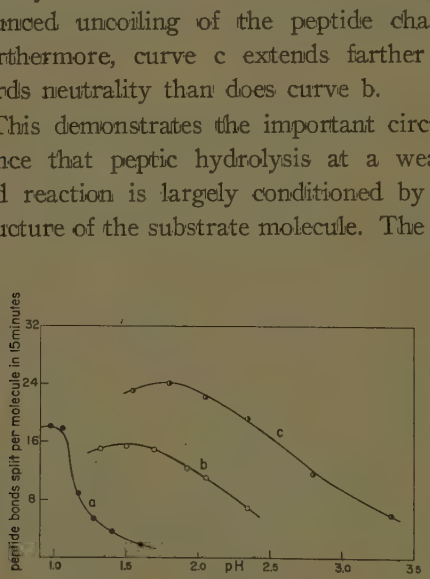

Fig. 2. Hydrolysis of egg albumin by crystalline pepsin (for explanation see text).
Curve a. Native egg albumin.
Curve b. Heat-denatured egg albumin.
Curve c. Urea-denatured egg albumin.

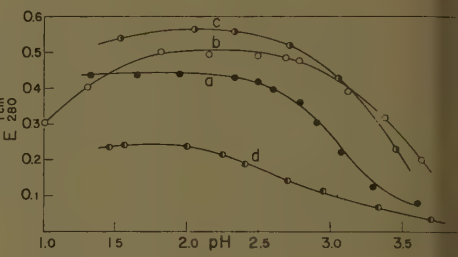

Fig. 3.

Curve a. (E_{280}^{1cm}). Hydrolysis of edestin by stalline pepsin.
Curve b. (E_{280}^{1cm}). Hydrolysis of edestin by gastric juice.
Curve c. (nonprotein nitrogen). Hydrolysis edestin by pepsin (Parke, Davis).
Curve d. ($E_{28'}^{1cm}$). Hydrolysis of heat-dena egg albumin by gastric juice.

that in some cases pepsin displays a siderable activity in the pH range ar 3.5 to 4 does not imply that the enzyme a catheptically active site; usually it indic that the substrate is in a denatured for

According to Baily (1942), edestin, w was employed as substrate in most of Bu experiments, denatures with great rap at pH values below 4. Hence proteo breakdown of this protein by pepsin inv a denatured substrate, and in accord the foregoing one would expect it t digested rather easily in the pH re 3 to 4.

That this holds true will be seen Fig. 3, curve a.

In addition, the author has studied p hydrolysis of denatured β-lactoglobulin casein, and of hemoglobin employing t different methods (Willstätter titra determination of nonprotein nitrogen, determination of aromatic amino acid

ichloracetic acid filtrate). In all cases
irves found show the same essential
es as do curve c, Fig. 2, and curve a,
. The curves have never shown the two
found by Buchs.
is the present study furnishes no
imental support of the assumption that
has a special catheptic function.

B. Does gastric juice possess a catheptic activity?

s problem has been approached in some
iments with edestin and with heat dena-
egg albumin as substrates (Fig. 3).

EXPERIMENTAL

ediately after withdrawal the gastric juice
luted with twice its volume of 0.5 M citrate-
uffer of pH 4, filtered and dialyzed against
d water.
tin (Roche) was dissolved in water with
n of 0.1 n HCl so as to form a solution
3.5 containing 3 g protein per 100 ml.
albumin was prepared according to Søren-
d crystallized four times. It was denatured
ting a solution in dilute HCl containing 4.4 g
0 ml, pH 3, at 100° C for 10 min. At this
alue the protein solution remains perfectly

reaction mixtures were buffered with
-HCl buffer according to Sørensen.
er digestion the protein was precipitated by
roacetic acid (final concentration 10 per
The filtrate was used for determination either
protein nitrogen or of its ultraviolet absorp-
t 280 mμ.
reaction mixtures corresponding to the
of Fig. 3 were composed as follows:

a. (UV absorption).
Edestin 1 per cent, crystalline pepsin (Armour) 0.077 per cent.
After 30 min. digestion at 30° C, 1 ml reaction mixture was added to 5 ml 12 per cent trichloroacetic acid at room temperature.

Curve b. (UV absorption).
Edestin 1 per cent, diluted gastric juice 0.2 ml per 3 ml reaction mixture.
After 30 min. digestion at 30° C, 1 ml reaction mixture was added to 5 ml 12 per cent trichloroacetic acid.

Curve c. (Determination of nonprotein nitrogen.)
Edestin 1.16 per cent, pepsin (Parke, Davis) 0.26 per cent. Digestion for 20 min. at 30° C.

Curve d. (UV absorption.)
Heat denatured egg albumin 2.2 per cent, diluted gastric juice 1 ml per 4 ml reaction mixture. After 50 min. digestion at 30° C, 1 ml reaction mixture was added to 7.5 ml 11.3 per cent trichloracetic acid.

It will be seen from Fig. 3 that the pH *vs.* activity curves of the protease of gastric juice are not fundamentally different from those of crystalline pepsin or Parke, Davis pepsin. Neither exhibits any trace of a peak in the interval from 3 to 3.5. In experiments where HCN was added in a concentration of 0.01 n to a reaction mixture corresponding to curve b, Fig. 3, no acceleration of the proteolysis was encountered.

These experiments do not support the assumption that gastric juice contains any catheptically active principle.

Of course this experimental material does not exclude the possibility that a strong peptic activity may veil a very weak catheptic activity, but if the latter exists at all it must be very slight.

SUMMARY

1) It has not been possible to confirm the hypothesis of Freudenberg and Buchs concerning the existence of a gastric cathepsin.

2) It is stressed that the structure of the protein substrate is the rate-determining fac-

tor for peptic digestion in the weakly acid region. The more extensively the substrate molecule is denatured (*i. e.*, the more its peptide chains are expanded) the more readily will it be hydrolyzed in this pH range.

3) In no case (even with five different substrates studied by three different methods) was any indication found of a two-peak pH *vs.* activity curve for proteolytic breakdown by pepsin or by gastric juice.

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E EXCRETION OF UREA BY THE HUMAN PAROTID GLAND

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ıe concentration of urea in the saliva first measured by Schöndorf in 1899, quantitative study on salivary excretion ea was not made until 1922 by Hench ldrich. These authors measured urea ammonia nitrogen in the saliva, in order oid the error introduced by a variable down of urea by bacterial ureases in d saliva. They found that the concen- n of urea plus ammonia in human mixed a was approximately 80 per cent of the ntration of urea in the blood. They ed further that salivary urea plus onia varied in proportion to the concen- n of urea in the blood and suggested the determination of "combined nitro- in mixed saliva may be used clinically e diagnosis of uremia. Later investiga- have chiefly been concerned with the tological problems related to the break- of urea by bacterial ureases in mixed a, but only few reports on the physiology ıe excretion of urea in the saliva are able. Updegraff & Lewis (1924) found urea plus ammonia nitrogen in mixed a of fifteen individuals averaged 76,1 ent of the urea nitrogen of the blood. Barnett & Bramkamp (1929) showed that the concentration of urea plus ammonia was greater in samples of parotid saliva secreted at slow than in those secreted at rapid rates. These authors and Bramkamp (1937), moreover, found that the concentration of ammonia in pure parotid secretion was always negligible.

The purpose of the present investigation was to examine the excretion of urea in human parotid saliva in relation to the rate of salivary secretion and the concentration of urea in the plasma. The results are compared to similar studies on the excretion of urea in the sweat, in the tears and in the pancreatic juice. A comparison of the excretion of urea in these four secretions indicates that a reabsorption of water occurs in the ducts of the parotid gland and the sweat gland whereas a similar mechanism of water reabsorption cannot be demonstrated in the lacrimal and pancreatic glands.

EXPERIMENTAL PROCEDURE

Subjects: 14 humans (8 men and 6 women) served as subjects for the experiments. 2 of them were healthy, 8 had minor diseases in the locomotor system and 4 had chronic renal disease with uremia. Most experiments were performed in the forenoon when the subjects were either fasting or had received a light "continental" breakfast only.

upported by a grant from "The King Chri- X Foundation".

However, in 8 experiments on 6 of the non-uremic subjects, urea was fed orally one hour prior to the test in amounts varying between 30 and 60 grams. By the examination of normals, of normals who had received urea orally and of uremic subjects, the salivary excretion of urea could thus be studied over a wide range of variation in the concentration of urea in the plasma (from 9.8 mg per 100 ml in one of the normals to 460 mg per 100 ml in one of the uremic subjects).

Stimulation of salivary secretion: Salivary flow was stimulated with lemon flavored candies or by the subcutaneous injection of pilocarpine hydrochloride (5—15 mg in isotonic saline). No differences associated with these two methods of stimulation were noted in the rate of excretion of urea in parotid saliva.

Collection of saliva: Parotid saliva was collected from the opening of Steensen's duct in small plastic cups by a method previously described (Thaysen, Thorn & Schwartz, 1954). Collections were made at accurately timed intervals of from 1 to 45 minutes duration in order to determine the secretory rate at various intensities of stimulation and under "resting conditions". Approximately 0.5 to 1.5 ml of saliva was collected in each period. The slowly secreted samples were collected in straight pipettes of 1 ml capacity, which were directly attached to the rubber tubing draining the collection unit. The rate of evaporation from such pipettes was measured and found to be negligible within 24 hours.

Venous blood was drawn in heparinized tubes at the beginning and at the end of each test or at least every hour in experiments of longer duration. The blood was immediately separated by centrifugation for the determination of urea in the plasma.

Determination of urea in plasma and in saliva was carried out by the colorimetric method of Archibald (1945). In two independent tests no demonstrable loss of urea occurred in pure parotid saliva which had been incubated for 24 hours at 37° C. All saliva samples in the present experiments were deproteinized and prepared for analysis by dilution and addition of sulfuric-phosphoric mixture within 2 hours of collection. The p bility of breakdown of urea by bacterial ur in the saliva was therefore considered t negligible.

The coefficients of variation for 10 r cate analyses for urea in saliva and in pla were 3.9 and 2.4 per cent respectively. F the data of these replicate analyses it ca calculated that a variation of about 5 cent may occur in the saliva/plasma centration ratio for urea merely due to lytical error.

RESULTS

The relationship between the excretio urea in the saliva and the rate of sali secretion was examined in 249 indiv saliva samples obtained in 25 experim on 14 persons with rates of salivary secr ranging from 0.016 to 2.35 ml per mi

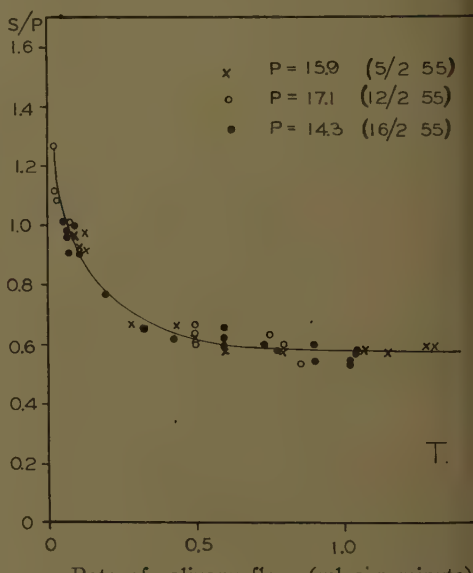

Fig. 1. The relationship between S_u/P_u and th of salivary secretion in a normal subje

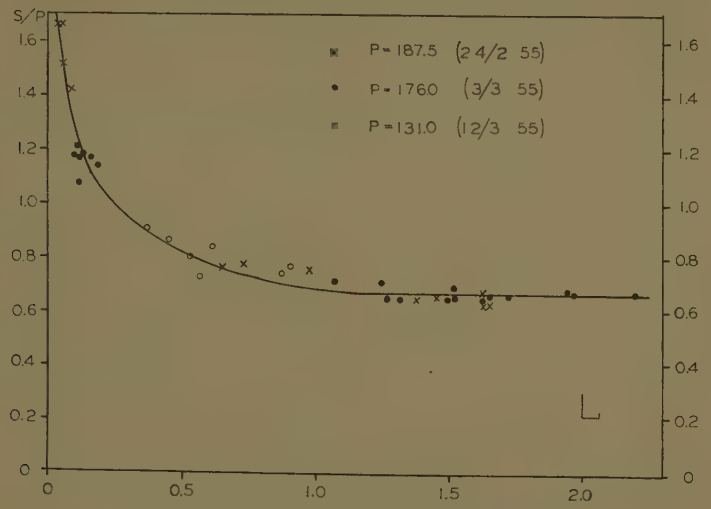

Fig. 2. The relationship between S_u/P_u and the rate of salivary secretion in a uremic subject.

s 1 and 2 show the results obtained experiments (82 individual saliva es) on 2 subjects with widely different ntrations of urea in the plasma. To ate comparison of the 6 experiments, ate of salivary secretion is plotted t the saliva/plasma concentration ratio ea, S_u/P_u, since it was found that the ntration of urea in the saliva varies portion to the concentration of urea in asma (see below). It will be seen that is approximately 0.6 and independent ner large variations in salivary flow at ory rates exceeding 0.5 ml per minute. retory rates below 0.5 ml per minute $_u/P_u$ ratio gradually rises and becomes r than unity at the low "resting" (un- ated) flows of saliva of about 0.02 to l per minute.

ne remaining 19 experiments, including dividual saliva samples, the "resting" ory rates were obtained in a few in- s only. The relationship between secretory rate and S_u/P_u was therefore not followed as closely as in the above 6 experiments. The experiments confirmed, however, that S_u/P_u is largely independent of secretory rate at salivary flows exceeding 0.5 ml per minute. Moreover, 47 samples obtained at secretory rates below 0.5 ml per minute all had S_u/P_u ratios greater than average of the remaining 120.

In 11 of the 25 experiments, stimulation of salivary secretion was commenced before application of the collection unit. This was done to facilitate application, since the opening of Steensen's duct was more readily visualized when it was dilatated by a brisk flow of saliva. In 14 experiments application of the collection unit was easier, and in these cases stimulation was not commenced till immediately after the collection unit had been placed in its proper position over Steensen's pore. In these experiments the first one (or two) samples of saliva, collected after stimulation of salivary flow, had a

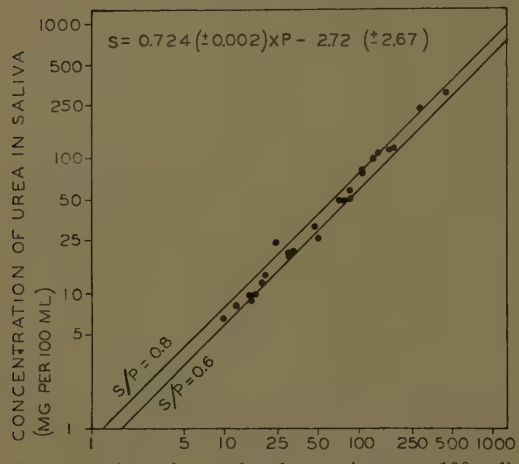

Fig. 3. The relationship between the concentration of urea in the saliva and the concentration of urea in the plasma (for details see text).

somewhat higher S_u/P_u ratio than the following, despite the fact that the secretory rates of the first samples always exceeded 0.5 ml per minute and were usually above 1.0 ml per minute. The higher S_u/P_u ratio of the initial samples is presumably due to the fact that the brisk flow of saliva produced by stimulation is mixed in the ducts of the gland with saliva produced at the low, "resting" secretory rates prior to stimulation.

Excluding 79 samples with secretory rates below 0.5 ml per minute and 16 "initial samples", the remaining 154 saliva samples from all 25 experiments had an average S_u/P_u ratio of 0.646 ($\pm$ 0.107). The coefficient of variation for S_u/P_u found experimentally (16.6 per cent) thus exceeded the coefficient of variation for S_u/P_u that might be expected from analytical error (5 per cent). An analysis of variance of S_u/P_u ratios from 5 subjects who had all been subjected to repeated tests showed that the value of F for the variance associated experiments on different subjects was critical value for F at the 1 per cent whereas the value of F for the variance ciated with experiments on different was below critical value for F at per cent level. From these results it concluded that the large variance of noted in the present experiments wa partly to physiological differences be persons and partly to analytical error the other hand no physiological diff could be detected between repeated e ments on any one person with the tech employed in the present study.

The relationship between the excret urea in the saliva and the concentrat urea in the plasma was studied in the 25 experiments as mentioned above. concentration of urea in the plasma 14 subjects ranged from 9.8 to 460 m 100 ml.

In Fig. 3 the concentration of u the plasma is plotted against the conc tion of urea in 154 individual saliva sa obtained at secretory rates exceeding per minute (excluding 16 "initial peri The points represent mean values of 2 to 12 determinations of the concent of urea in the saliva in each of the 25 e ments. Numerical analysis of the data the linear regression: $S = 0.724$ ($\pm$ $P - 2.72$ ($\pm$ 2.67). It will thus b that the concentration of urea in the sa secretory rates exceeding 0.5 ml per remains directly proportional to th centration of urea in the plasma over fold range of variation in the latter.

Furthermore, it was found that th in the concentration of urea in the observed at secretory rates below 0.5

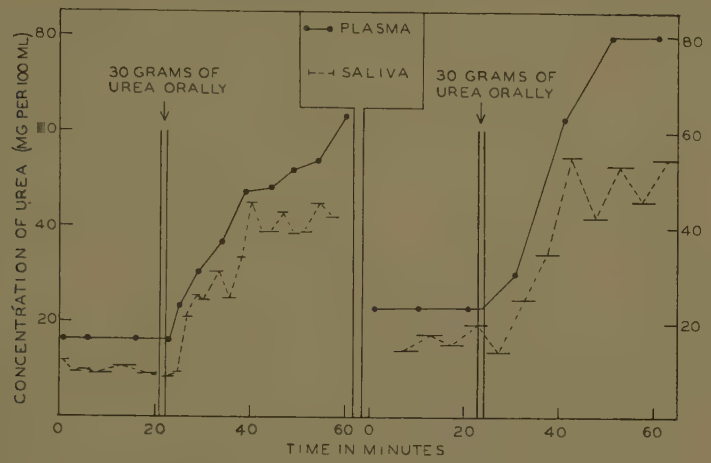

Fig. 4. The rise in the concentration of urea in plasma and in saliva, following oral ingestion of urea by two different subjects.

e, was unaffected by large variations concentration of urea in the plasma. fact is illustrated in Fig.s 1 and 2 show that the relationship between $_u/P_u$ ratio and the rate of salivary ion remains unchanged despite a 10- ifference in the concentration of urea plasma of the two subjects.

relationship between the excretion of n the saliva and a rising concentration a in the plasma was examined in 2 ments on 2 different subjects. The ntration of urea in plasma and in saliva etermined repeatedly before and after bjects had received 30 grams of urea The saliva was collected in syringes e collection unit was completely emp- t the end of each individual sampling lying suction to the syringe. This was in order to minimize the disturbing nce of the "dead space" of the unit. ry flow was stimulated with candies e secretory rate exceeded 0.5 ml per e in all samples collected.

The results are shown in Fig. 4. It will be seen that a rise in the concentration of urea in the plasma is followed by an almost simultaneous rise in the concentration of urea in the saliva. The ratio of the slopes for the rising concentration of urea in saliva and plasma is approximately equal to the S_u/P_u ratio at equilibrium.

The delay in the transfer of urea from plasma to the opening of Steensen's duct cannot be measured exactly in these experiments. From inspection of the two graphs it appears that the "appearance time" for urea in the saliva is about 1 to 2 minutes. Since salivary flow in both experiments was approximately 0.7 ml per minute in the first five minutes following ingestion of urea, the volume of the parotid gland should be between ¾ and 1½ ml. This is of course only a rough estimate because of the many approximations of the method. Moreover, no correction is included allowing for the time involved in the diffusion of urea from

the bloodstream to the lumen of the glandular acini.

It was attempted to determine the volume of the gland in another way using equation (1):

$$(1) \quad V = \frac{A}{C},$$

where V is the volume of the gland, A the amount of urea in the resting (unstimulated) gland and C the concentration of urea in the resting (unstimulated) secretion. C can be measured and A determined from equation (2):

$$(2) \quad A = V_0 \ (c_1 - c_2),$$

where V_0 is the total volume of the initial sample of saliva having a higher concentration of urea than the following, c_1 the concentration of urea in the initial sample and c_2 the average concentration of urea in all samples with flow exceeding 0.5 ml per minute, excluding the initial. The volume of the gland was determined in this manner in three individual experiments on the same subject and found to be 0.064, 0.179 and 0.077 ml. These values are considerably smaller than the values estimated from the "appearance time". This may be due to the fact that the latter method measures "resting" volume of the gland and the former "stimulated" volume.

DISCUSSION

In the present experiments the concentration of urea in the saliva is found to vary with the rate of salivary secretion. The saliva/plasma concentration ratio for urea, S_u/P_u appears to be independent of secretory rate and averages 0.65 at salivary flows exceeding 0.5 ml per minute. At lower secretory rates, however, S_u/P_u gra rises and becomes greater than unity unstimulated salivary secretion. A s observation has been made by Barn Bramkamp who found that the saliva/ concentration ratio for urea in parotid was on average 0.73 in "rapidly secr samples (0.52 to 3.00 ml per minute on average 0.95 in "slowly secreted" sa (0.10 to 0.58 ml per minute).

It is shown in the present investi that the concentration of urea in p saliva, collected at secretory rates exce 0.5 ml per minute, remains directly pr tional to the concentration of urea i plasma over a 46-fold range of variat the latter. This finding confirms the work of Hench & Aldrich and indicate urea is excreted in the saliva by a p of simple diffusion, and not *via* a s transport mechanism which could b saturated by increasing load. It is d strated, moreover, that large variatio the concentration of urea in the plas not affect the variation in S_u/P_u wi rate of salivary secretion. Fig.s 1 and strate that S_u/P_u becomes greater unity in the unstimulated secretion, a 10-fold range of variation in the c tration of urea in the plasma. This f indicates that the rise in S_u/P_u at low of salivary flow is not due to specific tion of a small amount of urea by the p epithelium in addition to the greater a excreted by simple diffusion. It is unlikely that the hypothetical sec mechanism should be so geared to th centration of urea in the plasma as to e always a constant fraction of the pre load at all rates of salivary flow.

ce it appears that one may, thus, de the possibility of an active secretion ea by the parotid gland it becomes of st to study the reason for the variation $/P_u$ with the rate of salivary flow. The interesting finding is that the concen- n of urea in the unstimulated secretion s exceeds that of the plasma. Dis- ding the possibility of an anomalous ion of urea through the parotid epi- m, the only possible explanation for phenomenon is that urea is raised to gher concentration in the saliva by reab- ion of water from a precursor solution ed within the secreting gland. The tory process should thus involve two ct steps: the formation of a precursor on in a region of the gland with a vely high permeability to urea and the orption of water from this solution in ion of the gland with a lower perme- y to urea. A variation in S_u/P_u with ate of salivary flow, resembling that ved in the present experiments, would if the rate of salivary flow were deter- d by the amount of precursor formed eas the reabsorption of water from the rsor solution remained essentially con- . The theory is forwarded that the tion of the precursor solution occurs glandular acini, whereas the reabsorp- f water takes place in the ducts of the . This assumption would fit well with ous studies on the excretion of urea by veat gland (Schwartz, Thaysen & Dole,) and on the excretion of sodium by parotid gland (Thaysen, Thorn & artz, 1954).

comparison between the excretion of in sweat (Schwartz et al.), in tears (Thaysen & Thorn, 1954), in pancreatic juice (Thaysen, Killmann & Bro Rasmussen, 1955) and in parotid saliva shows the following similarities and differences: Urea is excreted by simple diffusion in all four secretions. The secretion/plasma concentration ratios for urea are independent of secretory rate in sweat, tears and pancreatic juice. In parotid saliva the secretion/plasma concentration ratio varies in the manner outlined above. Only in sweat and in unstimulated parotid saliva does the secretion/plasma concentration ratio for urea exceed 1, and it has been suggested that a reabsorption of water from a precursor solution occurs within both glands. The secretion/plasma concentration ratio for urea is 1 or smaller than 1 in the tears and in the pancreatic juice at all secretory rates. Studies on the excretion of urea in these secretions do, thus, not permit any conclusions regarding the possibility of an internal circulation of water in the lacrimal and pancreatic glands.

SUMMARY

In 25 experiments on 14 humans the rate of excretion of urea in parotid saliva was measured in relation to the rate of salivary secretion and to the concentration of urea in the plasma. The ratio of the concentration of urea in the saliva to the concentration of urea in the plasma, S_u/P_u, averaged 0.645 at secretory rates exceeding 0.5 ml per minute. At lower secretory rates S_u/P_u gradually rose and became greater than unity at the "resting" (unstimulated) secretory rates of about 0.05 ml per minute. At all secretory rates S_u/P_u was independent of large variations in the concentration of urea in the plasma. It is concluded that urea is

excreted in the saliva by simple diffusion and not *via* a specific secretory mechanism that could become saturated by increasing load. The theory is forwarded that the secretion of parotid saliva involves two distinct steps: the formation of a precursor solution in amounts varying with the secretory activity of the gland and the reabsorption of an essentially constant amount of water from this precursor solution. A comparison tween the excretion of urea in sweat, s tears and pancreatic juice indicates th reabsorption of water occurs in the of the sweat gland and the parotid g whereas a similar mechanism of wate absorption cannot be demonstrated in lacrimal and pancreatic glands.

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THE PLASMINOGEN ACTIVATOR IN URINE AND THE URINARY TRYPSIN INHIBITOR

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fibrinolytic activity of normal urine cently found to be caused by an agent ing plasminogen, the precursor of the ytic enzyme of blood, Williams ; Astrup & Sterndorff (1952 a) ; Mohler, Jones, Dowdy & Guest . Urine also contains a substance inhibits trypsin, Dillard (1950). The ce in urine of varying amounts of this e inhibitor might interfere with esti- s of the plasminogen activator. It erefore decided to try to separate the nogen activator from the contami- inhibitor and to investigate each com- separately.

MATERIAL AND METHODS

ation of the plasminogen activator was ed with the standard fibrin plate method ly described, Astrup & Müllertz (1952). ine fibrin used contains large amounts of gen, which reacts with the urine activator plasmin and thus gives a measure of the of plasminogen activator.

in acts as an activator of plasminogen, ty, Ellis & Jensen (1952) ; Lewis & Fergu- 2) ; Astrup & Sterndorff (1952 b) ; Jacobs- 3). The action of trypsin on the standard ates is therefore complicated by formation in. The effect of the protease inhibitor in was therefore estimated on heated fibrin here the substrate is free from plasminogen, 1952). Standard fibrin plates were heated for 45 min. at 85°. Total destruction of plasminogen was verified by application of the plasminogen activator from urine.

The stock solution of trypsin contained 50 mg Armour crystalline bovine trypsin (containing less than 50 per cent $MgSO_4$) in 100 ml 0.05-N HCl and was stored in the ice box. The standard solution used in the estimations was prepared daily from the stock solution by diluting 10 times with 0.05-M diethylbarbiturate buffer containing 0.1-M NaCl (pH: 7.75; total ionic strength (μ): 0.15). Equal volumes of the trypsin solution and of serial dilutions of the inhibitor solution were mixed and the proteolytic activity estimated on the heated fibrin plates. By interpolation on a trypsin dilution curve the inhibitory effect could then be estimated. An example is presented in Fig. 1.

Bovine plasminogen was made from bovine fibrinogen (as prepared by Astrup & Müllertz (1952)) by defibrination with thrombin followed by ammonium sulphate precipitation, resolution in a small volume of H_2O, dialysis against 0.9 per cent NaCl and lyophilization. This procedure was adopted from that described before, Astrup & Sterndorff (1953).

Bovine plasmin was usually prepared by activation of plasminogen by minute amounts of trypsin followed by a heat treatment at pH 9.0 and 50° for 30 min. The resulting solution of plasmin was neutralized and lyophilized. Details of the interaction between plasminogen and trypsin will be published elsewhere. Samples of bovine plasmin were also prepared for control experiments by activation of plasminogen with the urine activator or by means of the soluble plasminogen activator isolated from pig heart tissue, Astrup & Stage (1952).

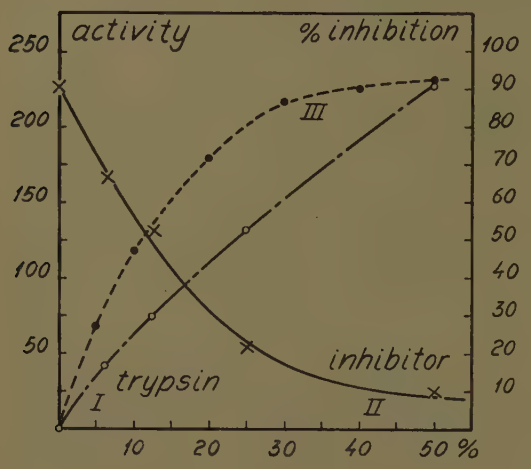


Fig. 1. *Effect of the urine inhibitor on trypsin. Trypsin curve (I): Abscissa:* Concentration of trypsin solution in percentages of the standard solution. *Ordinate* (left): Fibrinolytic activity. *Inhibitor curve* (II): *Abscissa:* Concentration of inhibitor in percentages of the undiluted inhibitor solution. *Ordinate* (left): Fibrinolytic activity of mixtures of the inhibitor dilutions with the standard trypsin solution. *Curve III:* Inhibition as obtained by interpolation of the activity of the trypsin inhibitor mixtures (curve II) on the trypsin curve (curve I). *Abscissa:* Concentration of inhibitor applied in percentages of the undiluted solution. *Ordinate* (right): Amount of trypsin neutralized in percentages of the trypsin control.

RESULTS

Preparations of the activator of plasminogen in human urine according to the method described previously, Astrup & Sterndorff (1952 a), sometimes produced a slight effect on heated fibrin plates indicating the presence in the preparations of small amounts of a fibrinolytic enzyme. Great care has to be used in the preparation of the heated fibrin plates to secure a fibrin substrate completely free from plasminogen in order to exclude the effect of agents activating plasminogen. Even after careful treatment of the plates a number of prepar still exerted a slight proteolytic effect. or dialysed urine did not normally pr lysis on the heated fibrin plates, even the effect on the standard fibrin plates taining plasminogen) was large, but sionally a sample of urine was rec which produced a slight effect on fibrin plates. These results indicate small amounts of fibrinolytic enzymes present occasionally in normal urine. effects could usually be observed only concentration and probable removal of fering inhibitory agents.

The inhibitory effect of normal uri trypsin was therefore studied, but only slight effects were observed. When hibitory effects of preparations of the u activator were investigated it was foun more or less inhibition of trypsin occ

It was also observed by comparing tion curves of different activator prepar that qualitative differences occurred be the preparations, and occasionally been found that dilution of the prepar produced enzymatic activity on the fibrin plates. The conclusion was that urine normally contains small am of inhibitory agents, and that these ar centrated together with the plasm activator in the process of preparation ously described. The trypsin inhibi present in normal urine in only amounts (Dillard, 1950).

1. *Separation of the plasminogen ac*

The separation of the plasminogen vator from the protease inhibitor pre difficulties. One of the reasons for that the activator is a very thermolabi

easily destroyed at acid reaction, p & Sterndorff (1952 a).

aration by means of isoelectric pre- ion or fractional precipitation with l or ammonium sulphate met with but success. Treatment of a concentrated tor solution (as prepared above) with ium phosphate or Zn (OH_2) removed tor as well as activator. It was found er that tricalcium phosphate at pH 7.6 n the presence of 0.01-M phosphate ed only the activator, while the in- r remained in solution. The activator again be eluted by means of 0.2-M hate. Such a procedure had been used fractionation of enzymes in seminal (Lundquist, Thorsteinsson & Buus, and Dr. Lundquist kindly informed out this procedure before its publica- The amounts of tricalcium phosphate d varied slightly from experiment to iment. Usually 60 mg per ml was the nt used. The final procedure adopted s follows:

sh human urine was filtered and made ne (pH 9, phenolphtalein paper) by ise addition of NaOH (~ 7-N solu- After centrifugation the supernatant eutralized (litmus paper) by addition N HCl. The solution was cooled in ice- and cautiously precipitated with 3 vol. cold ethanol. After 15 min. the pre- e was isolated by centrifugation, dis- in 0.9 per cent NaCl (one tenth of the al volume), and then dialysed overnight against 0.9 per cent NaCl in order to e excess of low molecular weight sub- s, which interfere with the subsequent ption process. A small sediment formed g dialysis was discarded. This con- centrated solution, containing the activator and the inhibitor, was adjusted to pH 7.6 and 0.2-M phosphate buffer (pH 7.6) was added to a final concentration of 0.01-M. The solution was shaken cautiously for 45 min. at room temperature with an optimal amount of tricalcium phosphate as estimated in preliminary experiments. After centrifugation the sediment contained the plasminogen activator, and the supernatant the inhibitor (see below). The sediment was washed once with 0.01-M phosphate (pH 7.6) and then stirred for 30 min. with an amount of 0.2-M phosphate (pH 7.6) corresponding to the original concentrated solution. After centrifugation the supernatant containing the activator was lyophilized. The yield usually amounted to 3.5 to 4.5 mg dry substance per ml urine, and to about 60 per cent of the original activity. The loss occurred almost exclusively during the alcohol precipitation.

2. *Preparation of the trypsin inhibitor*

Almost all the inhibitor was contained in the supernatant after the treatment with tricalcium phosphate (see above). The wash solution contained only small amounts and was discarded. Usually traces of activator were also present. The solution was therefore acidified to pH 3 with dilute HCl and heated for 30 min. in a boiling waterbath. This treatment destroyed the activator, but influenced the inhibitor very little. The solution was neutralized and dialysed against 0.9 per cent NaCl. This solution was stable when stored in the icebox with toluene, and it contained most of the original inhibitor activity in the urine. Dry preparations were also made by lyophilization.

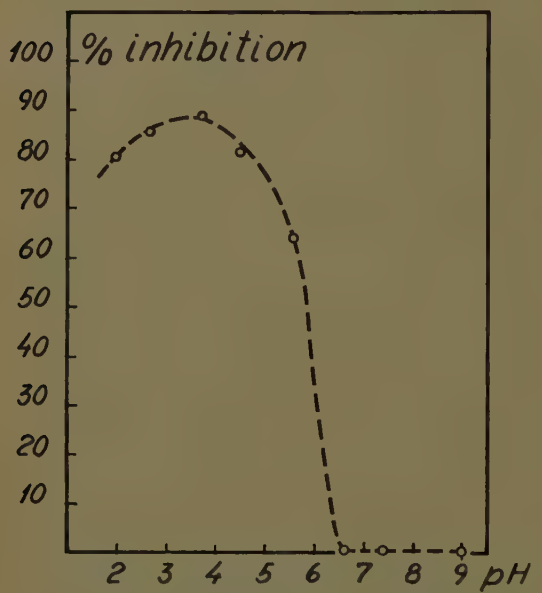


Fig. 2. *Thermostability of the trypsin inhibitor in urine. Abscissa:* pH during heating (30 min. at 100° C). *Ordinate:* Inhibitory activity recorded as decrease in trypsin activity in percentages of the activity of the trypsin control (compare Fig. 1).

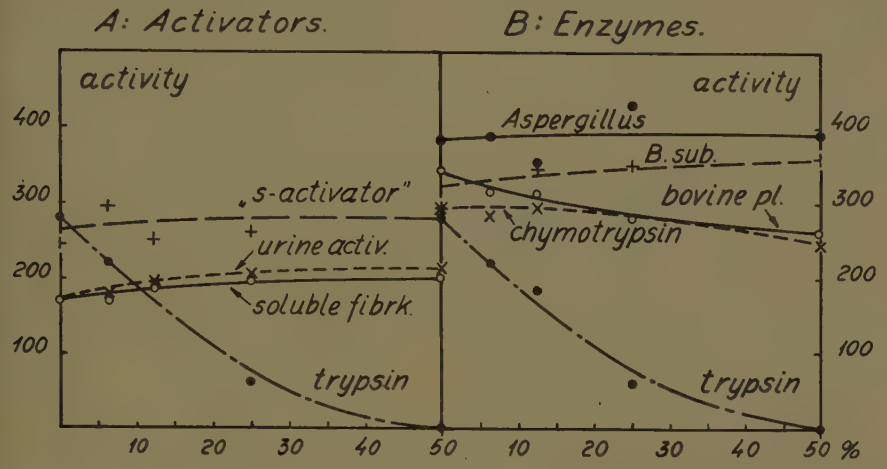
3. *Thermostability of the trypsin inhibitor*

The thermostability of the urine inhibitor was determined at different pH values. Solutions of the inhibitor were heated for 30 min. at 100° C and different pH values (1-N NaOH or HCl added). After neutralization and correction of the volumes with 0.9 per cent NaCl, aliquots were mixed with equal volumes of the standard trypsin solution and the resulting activity estimated on heated fibrin plates (Fig. 2). It is seen that the urine inhibitor is very stable at 100° C at pH 3 to 4. It is destroyed slowly at slightly acid reaction and rapidly at neutral and alkaline reaction.

4. *Effect of the trypsin inhibitor*

In urine the trypsin inhibitor ap together with a plasminogen activato was therefore of interest to study its on the fibrinolytic process. The conce tion of the plasminogen activator in uri normally so high that even dilute solu can be estimated (Bjerrehuus, 1952; A & Sterndorff, 1952 a). The concentrati the trypsin inhibitor is usually very lo can only be estimated in concentrated tion. Samples of pathological urine however yield deviating results and we confirm Dillards (1950) observation of concentrations of a trypsin inhibitor in p logical specimens. Though higher conce tions of the inhibitor interfered wit fibrinolytic agents, it could be shown the compound acts as a specific inh of trypsin.

In a series of experiments the inhi effects on a number of plasminogen activ were estimated on standard fibrin plates activator solutions had been previ diluted until they produced about effects on the plates. The following pl nogen activators were applied: trypsin urinary activator prepared as desc above; a water-soluble tissue activator pared from pigs heart (Astrup & 1952 a), a plasminogen activator pre from human blood by addition of st kinase (Müllertz & Lassen, 1953). inhibitor was prepared as described and the absence of any trace of plasmi activator was secured by appropriate trols. Traces of activator could be dest by repeating the heat treatment. Resu such an experiment are presented in Fig



3. *Action of the urine inhibitor on various proteases and plasminogen activators. Abscissa:* centration of inhibitor in percentages of the undiluted solution. *Ordinate:* Fibrinolytic activity tandard fibrin plates of a mixture of the enzyme or activator solution and the diluted inhibitor solu- Solutions tested: plasminogen activator from urine (urine activ.); streptokinase activated activa- from human blood ("s-activator"); soluble fibrinokinase; trypsin (2.5 mg/100 ml); chymotrypsin (7.5 mg/100 ml); bovine plasmin; Aspergillus protease (75 mg/100 ml); B. Subtilis protease (100 mg/100 ml).

seen that in concentrations of inhibitor e the effect of trypsin is completely pre- d no inhibitory effect on the activity of ther plasminogen activators is observed. rge amounts of plasmin are formed in ubstrate of the standard fibrin plates plasminogen activators are applied. effect of the inhibitor was also tested a number of proteolytic enzymes. They applied to standard fibrin plates in y equipotent solutions in order to be to compare with the results obtained the plasminogen activators. The fol- g enzymes were tested: trypsin, chymo- in (Armour), an Aspergillus protease wther & Lennox, 1950), a protease *B. subtilis* (kindly supplied by Prof. K. erstrøm-Lang, Carlsberg Laboratory, nhagen) and bovine plasmin prepared as described above. The results are presented in Fig. 3 B. It appears that the inhibitor also in this experiment acts preferably with trypsin. In the concentration applied there is a slight effect also on bovine plasmin and on chymotrypsin. These results with the proteases could be verified in experiments on heated fibrin plates, where no formation of plasmin takes place. The inhibition of trypsin presented in Fig. 1 took place on heated fibrin plates.

DISCUSSION

The experiments presented here show that human urine normally contains very large amounts of a plasminogen activator. Only occasionally are small amounts of a fibrinolytic enzyme also found. This enzyme is probably not trypsin, because in a single case studied the addition of cetylpyridinium

chloride inhibited its effect on heated fibrin plates. It had been found previously that cetylpyridinium chloride enhances the effect of trypsin (on normal fibrin plates), Astrup & Alkjærsig (1951). Presumably the enzyme is plasmin. Samples of urine containing small amounts of this enzyme might have been the cause of the proteolytic splitting of casein and albumin reported by some previous authors (Brodzki, 1907; Baumann, 1933; Buadze, 1935; Macfarlane & Pilling, 1947).

The presence in urine of ultraviolet absorbing compounds makes it difficult to use ultraviolet absorption in the estimation of proteolytic splitting. The preparation of plasminogen activator here described made it possible to use ultraviolet absorption (Hilger "Uvispek", 275 μ) and bovine plasminogen in a confirmation of the proteolytic splitting of casein containing human plasminogen as estimated by means of micro-Kjeldahl (Astrup & Sterndorff, 1952 a). Splitting of fibrin as followed by micro-Kjeldahl was used by Seelich, Pantlitschko & Kaiser (1953) in their investigations of the fibrinolytic agent in urine for which they found an activity optimum between pH 7.0 and 7.5. This corresponds to the observation by Macfarlane & Pilling (1947). v. Kaulla (1954) has recently prepared a purified preparation of the activating agent by means of adsorption to $BaSO_4$. Colgan, Gates & Miller (1952) used a fibrinogenolytic method in their estimation of fibrinolytic activity of urine in irradiated dogs. They assume that the urinary factor is identical with plasmin from the blood. Damgaard & Ungar (1952) using guinea pigs share this opinion.

Very little is known about the trypsin inhibitor in urine (for references see Dillard, 1950). The inhibitor as here prepared is a nondialysable and rather thermostable pound with a characteristic thermosta curve. The heat resistance had al been observed by Fujimoto (1918). very stable at acid reaction but is dest at neutral or alkaline reaction. This i the opposite of what was found for the vating agent in urine, but resembles the perties of a number of other pr inhibitors, see Laskowski & Lask (1954). The inhibitor has a very s effect towards trypsin (Fig. 3) and amounts are needed to interfere wit effect of plasmin, chymotrypsin or dif activators of plasminogen. Some e ments have shown the inhibitor to be pletely stable on shaking with chlor This is contrary to the trypsin inhibi blood, which is destroyed by chlor treatment. The inhibitor is present i small amounts in normal human urin effect could be demonstrated in the c trated preparations of the urinary acti The samples containing most inhibitor able completely to prevent the effect of volumes of a solution containing 2 Armour trypsin per liter. The prepar were concentrated 6—8 times relati fresh urine. In samples of pathological the inhibitor concentration can be higher.

SUMMARY

1. Normal human urine contains amounts of an activator of plasmi and small amounts of a trypsin inh Only occasionally were larger inh concentrations or traces of a prot enzyme encountered.
2. The plasminogen activator was sep from the trypsin inhibitor by adso on tricalcium phosphate.

ıe inhibitor has a strong effect on tryp-
ı. It reacts only slightly or not at all
ith a number of other proteases or plas-
inogen activators. It is rather heat
able at acid reaction but is destroyed
neutral or alkaline reaction.

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A METHOD FOR DETERMINATION OF FIBRIN IN PLAS

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Determination of fibrinogen in plasma fundamentally presents no great difficulties, and several suitable methods have been developed. Cullen & Van Slyke (1920) determined the quantity of fibrin formed by means of a standard Kjeldahl procedure, while Gram (1921) weighed the clot after defatting and drying.

Quick (1951) and Ratnoff & Menzie (1951) employed a method in which the fibrin was dissolved in sodium hydroxide; the protein content of the solution was determined by colorimetry after addition of Folin and Ciocalteu's phenol reagent. Parfentjev, Johnson & Cliffton (1953), Gerok (1953) and Jacox (1954) used nephelometric methods; they measured the turbidity produced on precipitation of the fibrinogen itself by addition of certain salts (Gerok, 1953; Parfentjev et al., 1953) or of a cationic detergent (Jacox, 1954).

Several of these methods are rather time-consuming, while others are not very accurate, so the author considers it justifiable to describe a new method, which is easy to use and at the same time very accurate, so that it is suitable for clinical as well as for scientific purposes.

THE PRESENT METHOD

The principle of the method.

It is a well-known fact that fibrin is paratively easily degraded by trypsin. author has utilized this fact: the fibri (adhering to a glass rod) is dissolve trypsin solution at pH 9. The protei tent of the solution is determined by me ment of its ultraviolet absorbancy at 2 where the aromatic amino acids ha absorbancy maximum.

The performance of the method

From the fasting patient blood is wit into a 2 ml conical centrifuge tube contain dry residue left after evaporation of 0.2 m 3.8 per cent tertiary sodium citrate solution. centrifugation 0.5 ml plasma is transferre siliconed test tube of 8.5 mm inside diamete the plasma is recalcified by addition of 1/40 M $CaCl_2$. The mixture is stirred wit 5 mm glass rod, the lower 2 to 3 cm of have been roughened, *e. g.* by treatment with fluoric acid. The rod is left in the tube 30 minutes.

The clot formed will adhere to the gla and by pressing it cautiously against the the tube it is possible to squeeze practic

out of the clot, which remains as a film ing the lower part of the rod. Now the clot ised with physiological saline and left sub- ed in physiological saline, which is changed at interwals of ca. 30 minutes and twice at als of ca. 1 hour.

is treatment will have removed contaminating proteins, and now the glass rod is trans- to a small test tube with a circular mark nl and containing ca. 1 ml 0.1 n NH_4Cl — NH_3 of pH 9. To this is added 0.125 ml of a solu- containing ca. 50 mg trypsin in 20 ml 0.0025 n Upon incubation for one hour at room temper- or for 30 minutes at 37° C the clot will as a e completely digested. The glass rod is raised the liquid surface and rinsed with buffer, he volume is made up to 5 ml. The absorbancy 0 mμ of the resulting solution is measured, containing the same amount of trypsin being as blank. The absorbancy of this control on against the buffer as blank was 0.120. The r used 10 mm silica cells and a slit width of m.

naller amounts of plasma may very well be without loss of accuracy. For example, nay employ only 0.2 ml plasma and 0.050 ml e trypsin solution. In this case it is advisable the clotting take place in a tube of 6.5 mm al diameter and to use a glass rod of ca. m.

e washing procedure may be shortened to 30 minutes without serious loss of accuracy, cularly when 0.2 ml plasma sample are used. is way the entire determination can be done out 90 minutes, which may be desirable for n clinical purposes.

Details of the method

hen tubes and rods of the dimensions d are employed, a fibrin film of suitable th and thickness are obtained. Other- it is recommended to check wether the ing procedure is sufficient to remove erum protein. This may be done by adding an equal volume of 20 per cent trichloracetic acid to a sample of the last washings.

The solution obtained after digestion of the clot has a slight opalescence caused by plasma lipids adsorbed to the fibrin. If the blood sample has been withdrawn from a fasting patient the error caused by the opalescence is insignificant, *cf.* the fit of the single points to the calibration curve (Fig. 1). In pathological cases of lipemia, or if the blood sample originates from a nonfasting patient, the sample and the blank must be shaken with ether before being read in the photometer if a highly accurate result is desired. For most clinical purposes this will be unnecessary.

The trypsin used was a commercial, crystalline product, "Trypure", prepared by "Novo terapeutisk Laboratorium", Copenhagen. It is furnished in vials containing 50 mg at a price of ten shillings, sufficient for ca. 160 determinations.

The ammonia buffer is prepared by mixing 420 ml 0.1 M NH_3 with 580 ml 0.1 M NH_4Cl.

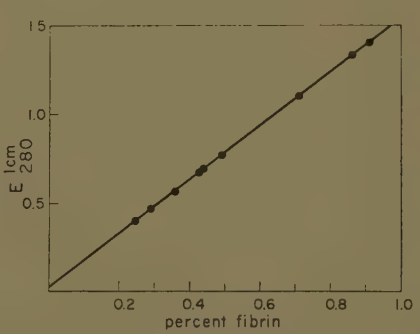

Fig. 1. Calibration curve for samples of 0.5 ml plasma.

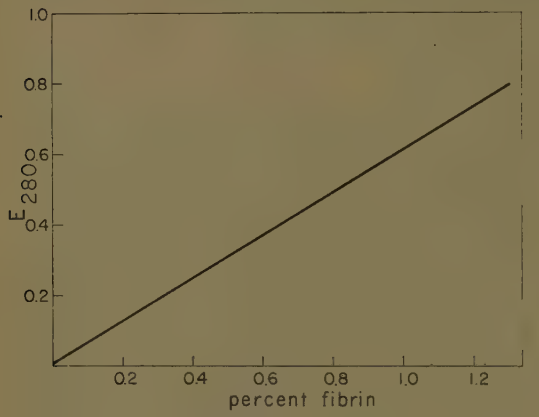

Fig. 2. Calibration curve for samples of 0.2 ml plasma.

The calibration curve

The calibration curve was determined by simultaneous quintuplicate photometric determinations and quintuplicate Kjeldahl analyses of fibrin from plasma derived from 9 patients. The nitrogen content of each sample was converted into per cent protein on the basis of Bailey's statement (1944) that fibrin contains 17.1 per cent N.

The curve is shown in Fig. 1. An absorbency of 1.00 indicates a fibrin content of 0.645 per cent. The curve is a straight line intercepting the axis of ordinates 0.020 units above the origin. This is due to the slight opacity caused by the fat content of the clot.

When the determination is performed on a plasma volume of 0.2 ml the result may be read from the curve in Fig. 2. This curve intersects the ordinate 0.008 units above the origin; an absorbancy of 0.400 indicates a fibrin content of 0.645 per cent.

Table I.

No.	$E_{280}^{1\ cm}$
1	0.555
2	0.557
3	0.557
4	0.557
5	0.559
6	0.557
7	0.560
8	0.553
9	0.560

The accuracy of the method

Table I shows the results of 9 determ tions in the same plasma. The accura seen to be high, the greatest deviation the mean being 0.5 per cent of the value.

A source of error is encountered i cases where the plasma has a spontan fibrinolytic activity. If this is suspected content of fibrin may be determined afte clot has been left in contact with the s for 30 minutes (as in the present meth for one hour, and for two hours. The time value may be found by extrapol

SUMMARY

A method for determination of pl fibrinogen is described. The fibrin cl dissolved with the aid of trypsin, and protein content of the resulting soluti determined by ultraviolet photometry. method is accurate as well as convenie is suitable for clinical and scientific pur including studies of the process of fib lysis. A determination can be perfo upon a plasma volume of 0.5 ml or 0.

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THE ELECTROPHORETIC PATTERN OF PROSTATIC LIPID SECRETION IN NORMAL AND PATHOLOGICAL CONDITIONS

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The present author has earlier demonstrated that, by means of a paper electrophoretic technique, the proteins of the normal prostatic secretion of Man can be separated into three clearly defined fractions, the migratory characteristics of which are identical with those of blood serum α-, β-, and γ-globulins.

The quantitative distribution of the protein fractions was on the whole the same in all the cases in a urogenitally healthy material. However, in certain specific pathological conditions of the gland, the quantitative distribution of the protein fractions was altered in a significant way characteristic of the morbid condition in question (Nylander, 1955).

The lipid content of the prostatic secretion in Man was analysed qualitatively as well as quantitatively by Moore, Miller & McLellan (1941) and Scott (1945). Also Iversen (1953) presented a report of his determinations of the total lipid content of prostatic secretion in man, relating it to the acid phosphatase activity of the secretion. An examination of the results arrived at by the above authors shows that the lipid content in the secretion of the prostate gland is subject to marked variations, evidently not related to age nor, according to Iversen, to the phosphatase activity of the secretion. M *et al.* (1941) give a mean value of 940 m cent, Scott 286 mg per cent, and Iverse mg per cent. According to the two first tioned authors, the content of total esterol seems to constitute less than 2 cent of the total lipid content of the secr Apparently, no attempts have been ma analyse more closely the lipid constit of the prostatic secretion, nor to place quantitative distribution in any relati the different pathologic conditions i gland. In view of the characteristic el phoretic patterns of prostatic secretion teins, ascertained in normal and in v morbid conditions in the gland and re to above (Nylander, 1955), an attem separate any lipid fractions in the sec from normal and pathologically changed static glands by means of a paper el phoretic technique seems indicated.

MATERIAL AND METHOD

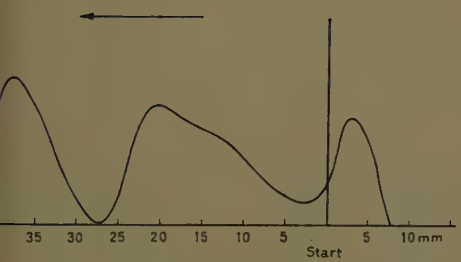
In the present investigation a clinical mat used that has been accounted for in an earlier (Nylander, 1955). Briefly, it consists of 28 of urogenitally healthy men, 19 cases of prostatic hypertrophy and 11 cases of chroni statitis.

paper electrophoretic technique employed so the same as that in the work referred to exept that 0.5 ml of secretion, instead of was applied at the start.

lipid material contained in the filter paper vas stained with a solution of Sudan black ing to Swahn (1953). By colorimetric deter- ons of each 5 mm of the paper strip, a n could be drawn up by plotting in a coor- system the extinctions against the distance ed.

RESULTS

. 1 illustrates the characteristic lipid n obtained by electrophoresis of a nor- rostatic secretion. It emerges that the content of the normal prostatic secre- is relatively small (low extinctions). components are discernible. The first represents the lipid fraction with the st rate of migration. To judge from its on in the diagram, this fraction seems respond to Fraction II of the prostatic ion proteins with the same rate of migration as the β-globulins in the blood serum. The second peak, which is broader and gives the impression of being composed of two, corresponds to Fraction III of the prostatic secretion proteins and, accordingly, to the γ-globulins of the normal blood serum. Finally, at the starting-line and behind it a third component is found which does not migrate at all in an electric field. The diagram presented in Fig. 1 is ideal in so far as the separation there is more pronounced than in any other determination. In several cases these three peaks can only be discerned with difficulty. It is therefore hard to establish, with any degree of certainty, an electrophoretic pattern of lipids typical of the normal prostatic secretion.



A lipid diagram obtained by paper electro- horesis of normal prostatic secretion.

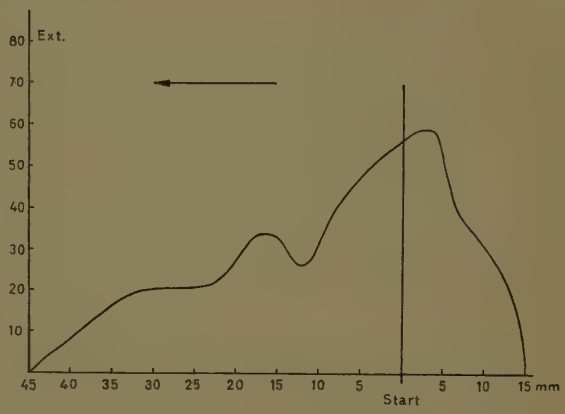


Fig. 2. Representative lipid diagram from a case of benign hypertrophy of the prostate.

In benign hypertrophy of the gland, as shown in Fig. 2, the appearance of the diagram changes in a characteristic manner. To begin with, it is evident that the exprimate obtained from the hypertrophic prostate contains more lipid material than that of the normal gland. Secondly, the same three frac-

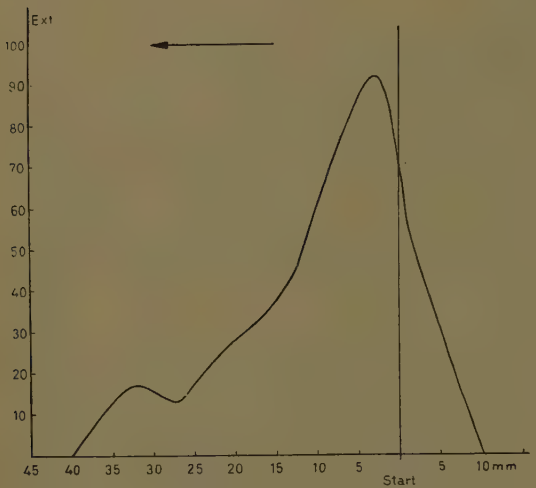


Fig. 3. Lipid diagram from a case of chronic prostatitis.

tions are obtained though with the difference that the fraction close to the starting-line is extremely increased. This occurs throughout in the cases of benign hypertrophy. It also happens in the cases of chronic prostatitis. It therefore seems impossible, in the present authors opinion, to distinguish this diagram from that of benign prostatic hypertrophy (cp. Fig. 3).

DISCUSSION

It has earlier been shown that the prosatic gland changes its secretory activity qualitatively as regards the protein constituents in different pathological conditions, *e. g.*, benign prostatic hypertrophy, chronic prostatitis and carcinoma of the prostate. This evidently occurs also with regard to the lipid components of the secretory product of the gland.

In prostatic hyperthophy and chronic pr titis the lipid content seems to be incre which applies throughout to the fra situated at the starting-line in particular. fraction is probably composed of c microns. The other two fractions obs seem to have the same rate of migrati the β- and γ-globulins and probably c of β- and γ-lipoproteins or lipid subst with the same rate of migration as lipoproteins. However, it should be po out that the identification of the lipid tions in the normal prostatic secretion difficult in view of the faintly coloured strips obtained from the normal pro secretion.

SUMMARY

The electrophoretic pattern of pro secretion lipids was examined by mea a paper electrophoretic method. The c material included healthy subjects and sons suffering from benign prostatic h trophy and chronic prostatitis. Three fractions could be identified in the n prostatic secretion, though the lipid c was rather low judging from the faint c intensity of the paper strips.

In prostatic hypertrophy and chroni statitis, the lipid content of the paper was considerably increased. Above a fraction lying close to the starting-lin increased.

The changed secretory activity of the in pathologic conditions, with special r to the lipid content, is pointed out.

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A METHOD OF ESTIMATING THE ACTIVITY AND ELECTROPHORETIC PROPERTIES OF 'ACID' PROSTATE PHOSPHATASE

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In 1935, Kutscher & Wolbergs demonstrated that the prostatic secretion in man contains a very high concentration of the enzyme "acid" phosphatase. Gutman & Gutman (1939) were able to report that its production was influenced by the androgenic hormones. In a comprehensive study on the same subject From Hansen (1949) registered the same close relation between the acid phosphatase activity of prostatic secretion and the production of testis hormone in man.

As a rule, the acid phosphatase activity is chemically determined according to Gutman & Gutman (1938), or by a modification of their method. The acid phosphatase activity is expressed as a function of the hydrolysis of a buffer-substrate system. (Example: disodium monophenyl phosphate is hydrolyzed by the enzyme activity and the split phenol is colorimetrically determined. The enzyme activity is thus expressed as a given function of the phenol separated from the system during a given period of time.) Histochemically, the acid phospatase activity is demonstrated according to Gomori (1941) by incubating the histological section in a lead sodium glycerophosphate solution. By immersing in yellow ammonium sulfide, the lead split by hydrolysis is perceived as dark deposits of lead sulfide, the quantities which are used as a measure of enz activity.

Earlier, the present author studied the tein and lipid components of prostatic s tion by means of a paper electropho technique. (Nylander, 1955 a & b.) In o to determine, if possible, the acid p phatase activity and the electrophoretic perties of the enzyme — or of the compo to which it might be bound — I have to subject the filter paper strips run in electrophoretic procedure to the formenti histochemical method of analysis.

Prostatic exprimates were obtained fro healthy subjects and 2) persons suffering benign prostatic hypertrophy. The electroph technic was principally in accordance with tha scribed by Detker & Andurén (1954). 1 mm³ secretion was run in the electrophoretic pr When the electrophoresis was terminated, the was dried at room temperature and immers cold acetone for 2 hours for fixation of the pro After drying the paper was submitted to Go histochemical method. Using a buffer soluti pH 8.6 in the electrophoresis, only a very faint of the precipitated lead sulfide indicative of en activity was obtained as a result of the chemical treatment, though it was originally posed that a reactivation of the enzyme ac would take place at the optimal pH of 4.5 substrate solution. By using a Gomori buff a pH of 4.5 in the electrophoresis, a very

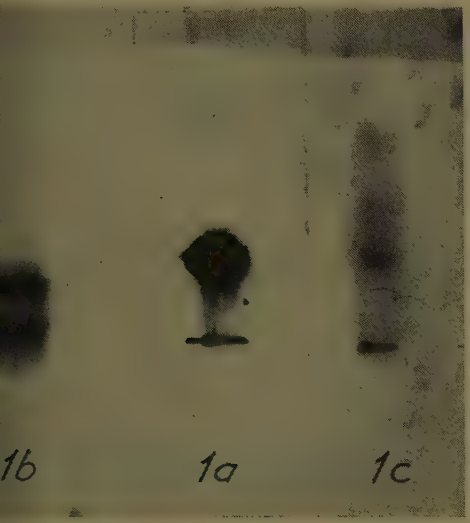

Fig. 1. Secretion collected from a normal prostate gland.

Paper electrophoresis run at a pH of 4.5. The acid prostate phosphatase' activity is indicated by the heavy spot of lead sulfide precipitate. The same sample of secretion run simultaneously in the paper electrophoresis procedure and stained for proteins. It is seen that the very faint fraction with the highest rate of migration corresponds to the spot of enzyme activity (1 a).

The protein pattern of the same sample of secretion but here the paper electrophoresis was run at a pH of 8.6. Three well defined protein fractions are visible.

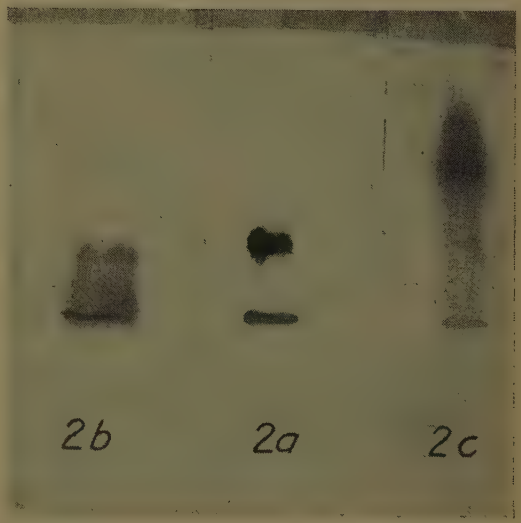

Fig. 2. Secretion collected from a case of benign prostatic hypertrophy.

2a: Paper electrophoresis run at a pH of 4.5. The paper strip is 'histochemically' stained for 'acid phosphatase' activity. Note the rather small spot of lead sulfide precipitate as an indication of the enzyme activity.

2b: The same sample run simultaneously in the paper electrophoresis but stained for proteins. Note the rather distinct protein fraction having the highest rate of migration and corresponding to the lead sulfide spot of fig. 2b.

2c: The protein pattern of the same sample. Here the paper electrophoresis is run at a pH of 8.6.

pitation of lead sulfide was produced as a of the histochemical treatment of the paper. om Fig. 1 a, it may be seen that the sulfide precipitate indicative of enzyme ity is found on a spot corresponding to irst faint protein fraction of the normal atic secretion (Fig. 1 b), here stained proteins and run simultaneously and arly in all other respects. This protein ion has earlier been described by the nt author and shown to correspond to blood serum α-globulins with regard to the migratory properties in the electric field. The electrophoretic pattern of prostatic secretion proteins run at a pH of 8.6 is shown to the right (Fig. 1 c).

Fig. 2 illustrates the prostatic secretion from a case of benign prostatic hypertrophy. In this instance the enzyme activity is much less pronounced (Fig. 2 a). The rate of migration is the same, corresponding to the

first protein fraction of the secretion (Fig. 2 b). As was earlier pointed out (Nylander, 1955a), this protein fraction is more strongly coloured in secretions of hypertrophic prostates, as evident from Fig. 2 c showing the electrophoretic pattern of the proteins run at a pH of 8.6.

Accordingly, by applying a drop of prostatic secretion to a strip of filter paper and running a paper electrophoresis at a pH that is optimal for the acid prostate phosphatase activity (4.5), this activity can be reproduced on the paper by means of a histochemical technique, involving incubation in a buffer-substrate solution. Thus, by this method it is possible 1) to determine the relative amount of enzyme activity, and 2) to establish the migratory characteristics of the enzyme, which is a globulin, in the electric field.

SUMMARY

By combining a paper electrophoretic and histochemical technique a method was obtained for ascertaining the relative activity and the electrophoretic properties of the acid prostate phosphatase.

It was demonstrated that in benign static hyperthropy the enzyme activity considerably diminished.

It was also ascertained that the enz which is a globulin, has the same migra characteristics as the blood serum α-gl lins.

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HE SERUM LIPOPROTEINS IN SLIGHT MALNUTRITION

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the investigation of the lipoprotein pat- n human sera attention has been focused y on diseases often accompanied by osclerosis. The lipid partition between arious protein fractions has been deter- l by flotation in the ultracentrifuge, by ol fraction according to Cohn et al. y electrophoresis in a filter paper im. In most diseases studied an in- e has been found in the normal or patho- l β-lipoproteins. Little is known of echanisms determining the partition of pids between the α and β-lipoproteins. known from the work of Barr, Russ ler (1953) and of the Gofman group) that in young women the level of -lipoproteins is higher than in young and that estrogen therapy produces a relative and an absolute increase of lipoproteins. It thus seems reasonable sume that the synthesis — at least of -lipoproteins — is influenced by hor- secreting organs. It is as yet not n whether the α- and β-lipoproteins ormed and metabolized independently h other or not.

TECHNIQUE

lipoproteins were separated and colored by ethod of Swahn (1953). However, since it ly desired to separate the α group from the β group, a shorter separation was considered sufficient. The albumin fraction and α-lipoproteins were thus not separated from one another, but calculated as a single fraction — the α group. An advantage of the shorter migration distance is that it diminishes the error due to adsorption of lipoproteins to the paper. The albumin frontier is allowed to migrate 6 cm.

MATERIAL

Blood specimens from 50 registered blood donors in the fasting state. None of the sera showed turbidity suggestive of postalimentary lipaemia.

Twenty-seven persons who had within the last five years undergone partial gastrectomy. Some of them reported that they felt well, while others persistent gastric trouble and loss of weight.

RESULTS

Total serum protein was analyzed in sera from 20 of the pathologic cases. The mean value found was 7.0 per cent. Electrophoretic analysis of albumin showed a mean value of 4.9 per cent against our normal value 4.7 per cent with S.D. $\pm$ 0.3. The values found speak against a pronounced malnutrition since the albumin values decrease relatively early in protein deficiency.

It is clear from the table that the concentrations of total lipid and phospolipid were lower in the group with slight malnutrition. The quotient between the α-lipoproteins and

Table *showing the variation of some lipid variables in a healthy (H) group and* a group with slight malnutrition.

	Total lipids mg per 100 ml		α-lipoprotein in per cent of total lipids		α-lipoprotein lipids mg per 100 ml		Phospholipid mg per 100	
	H.	Maln.	H.	Maln.	H.	Maln.	H.	M
Mean value ..	710	530	30	39	210	213	240	1
ε	12	32	0.4	1.6	3.7	13.2	5.4	
S.D. (σ) .	108	136	3.8	8.1	33	56	27	
Number	79	18	79	27	79	18	25	

the total lipids was, however, statistically significantly lower in the healthy group. The two groups however did not differ with respect to the concentration of the α-lipoproteins. This implies that the difference found between the two groups was due to the decrease in the β-lipoproteins. This suggests that the concentration of the α-lipoproteins and of the β-lipoproteins are determined by separate mechanisms and that the concentration of the β-lipoproteins but not of the α-lipoproteins varies with the nutritional state.

The lipoprotein pattern in obesity will be the subject of a later paper.

SUMMARY

In slight malnutrition the values for lipids, phospholipids and β-lipoprotei crease. The concentration of α-lipopr remains unchanged.

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NEW METHOD FOR DETERMINATION OF BACTERIAL SENSITIVITY TO CHEMOTHERAPEUTIC AGENTS BY MEANS OF PAPER STRIPS

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(Received for publication May 27, 1955)

e need for rapid and simple methods etermination of bacterial resistance to ances used in chemotherapy has in- ed parallel with the introduction of new iotics. Not so many years ago, dilution ods were those most frequently used, acteria being cultivated in solid or liquid a containing antibiotic substances in n concentrations. The sensitivity of acteria is then obtained by reading the mal inhibiting concentration. In the Bacteriological Laboratory such a od has been in use for many years ing & Wallmark, 1951). These methods ery reliable and are still much used for al purposes. For routine tests, however, have proved to be too time-consuming result of the many substances a deter- tion must now include. It has therefore necessary to use more rapid methods utine work. Diffusion methods have n themselves to be of great value in cases. By these methods the antibiotics dsorbed to paper disks and the resi- e of bacteria is determined by measuring ones of inhibition that appear when the ria are grown on agar plates prepared these disks. Such diffusion methods been described by many authors (Erics- Högman & Wickman, 1954; Erlands- 1951).

In this paper, we present a report on the determination of bacterial resistance to antibiotics by means of a new diffusion method. This method is, in our opinion, superior in some respects to the methods using paper disks.

The aim of our investigation was to work out a rapid and simple method that would require only small quantities of media and at the same time ensure satisfactory accuracy. From the very beginning, therefore, we endeavoured to find a method capable of fulfilling the following conditions: 1) one agar plate (normal size) for each determination, and 2) a reference bacterial strain, with known resistance to the antibotics used in the test, to be included as a standard. In this way, it would be possible to correct for many errors due, for example, to variations in media, differences in the manual performance of the tests, and instability of the antibotics. Correction for such variations seems to us to be of great importance for a satisfactory determination.

METHOD

In the method finally evolved we used a form of "test strips" manufactured in a special way. The strips (Fig. 1) consist of filter paper impregnated with sulfathiazole plus five of the most commonly used antibiotics. The substances ad-

Fig. 1. Test strip. The white rectangles (1—6) contain the following substance: 1. Sulfatiazole. 2. Penicillin. 3. Chlortetracycline. 4. Oxytetracycline. 5. Chloramphenicol. 6. Streptomycin.

sorbed in the white rectangles in the strip are separated from each other by zones impregnated with linseed-oil (see below). In this way diffusion of antibiotics within the strip is effectively prevented. One side of the strip is marked with a black line. Starting from this line the substances come in the following order: sulfathiazole, penicillin, chlortetracycline, oxytetracycline, chloramphenicol and streptomycin.

The principle for the use of the strips is the following. The strip is placed on an agar plate (peptone-free blood agar is a suitable substrate). One-half of the plate on one side of the strip is smeared with a suspension of the test bacterium and the other half is smeared in a similar way with a suspension of the standard strain. The bacteria are usually diluted 1/100—1/1000 with saline if they are obtained from a broth culture. As standard strain we have used *Staph. aureus* 209-P. The plates are incubated for 18 hours at 37° C. Then the zones of inhibition (Figures 2 and 3) are measured for both the test and the standard bacterium. The degree of resistance is calculated from these measurements. A careful analysis of the pattern of inhibition makes it also possible to establish when two adjacent antibiotics exert a synergistic or antagonistic effect on a bacterial strain.

The concentrations of the substances used in the strip are chosen on the basis of experiments so that the zones of inhibition for the standard strain will amount to about 10 mm. Each rectangle contains the following amounts of substances: sulfathiazole 0.1 mg, penicillin 1 I. E., chlortetracycline 10 γ, oxytetracycline 10 γ, chloramphenicol 20 γ, and streptomycin 50 γ.

COMMENTS

The degree of resistance, that is to say, if the isolated strains are to be characterized as "sensitive", "fairly sensitive", or "resistant", is calculated by comparing the of the inhibition zones obtained for tes reference strain respectively. With method, the degree of resistance is base on the absolute value, in mm, of the i tion zone but on the relation between zones, one belonging to the test strain to the standard strain. As the absolute of the inhibition zones can change fro to day with the same strain, it is po to diminish the errors by computin results in this way.

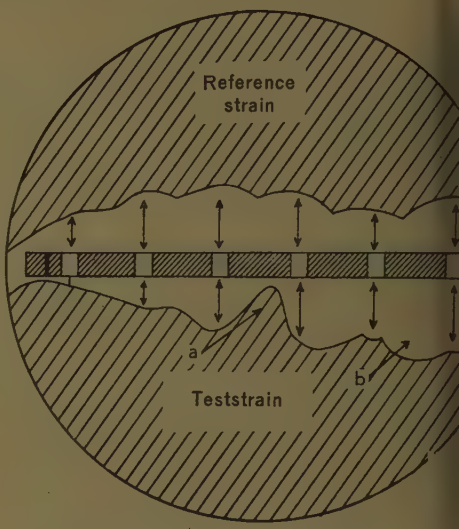

Fig. 2. Test strip with inhibition zones on agar plate.
↕ Size of inhibition. The arrows a and b cate antagonism and synergism between tw biotics.

The first strips were hand-made. Thi rather laborious and time-consuming, t impregnation of the filter paper, in ticular, involving much work. We ther approached the Graphical Research La tory in Stockholm (Messrs. I. Olss L. Pihl) and the Laboratory of the Sw Pharmaceutical Association (Mr. I.

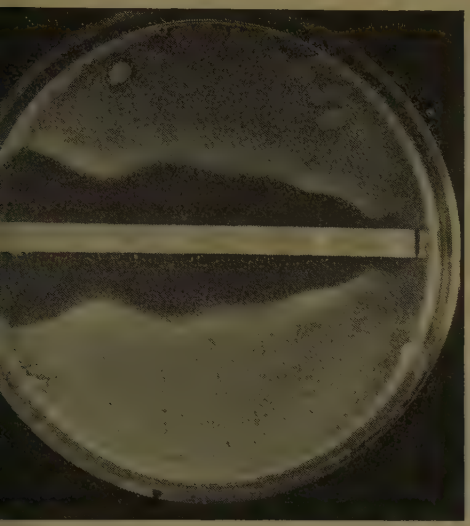

Fig. 3. Test strip with inhibition zones on agar plate.

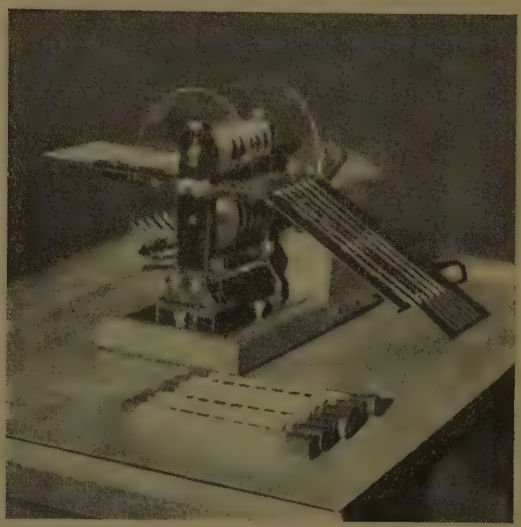

Fig. 4. Machine for impregnation of the strips with antibiotics (see text).

) with a view to solving these problems. collaboration has resulted in many tech- improvements, and a special machine constructed for the application of the iotics on the strips. To give an idea he method used now, the following int is presented.

n a number of pieces of blotting-paper a d-striped pattern is printed with a speci- prepared linseed-oil varnish that serves locking zones between the antibiotics. sheets are then cut to a definite size × 85 mm). The unprinted zones be- n the printed bands are impregnated different solutions of antibiotics. After g, the sheets are cut into strips perpen- ar to the impregnated bands.

special machine has been constructed ne impregnation of the unprinted zones antibiotic solutions (Fig. 4). This ine consists of a stainless steel chest ed into several smaller partitions into n the antibiotic solutions are poured. In each of these partitions a metal disk hangs down. All the disks are attached to a spindle, and there is a constant distance between the disks corresponding to the unimpregnated bands on the filter paper sheets. When the disks rotate, they take up a constant amount of the solutions. Against the upper side of each disk there is a counter pressure disk of the same kind. The strips are impregnated with antibiotics by feeding the filter paper sheets between the printing disks and the counter pressure disks. The printing surface of the disks is milled in order to obtain better wetting and greater accuracy in the amount of solution absorbed. By means of an automatic system regulating the flow of antibiotic solutions to the chests the same level is maintained. This is important because the amount of liquid absorbed depends on this level.

It is also possible to control the absorption of liquid by regulating the rotation speed of the disks. Attention must also be

paid to the quality of the filter paper used. We have used a special type of blotting-paper composed of 50 per cent rags and 50 per cent paper pulp .

The construction of the antibiotic printing-press is such that the motor can be readily uncoupled and the other sections sterilized in an autoclave. For this reason all details are brightly chromium-plated. To ensure protection against dust, and so on, during the printing of the sheets a plexi-glass cover is placed over the printing surfaces. After having passed the machine, the sheets with the absorbed antibiotic solutions are taken up on a perforated table that is coupled together with the machine. The sheets are dried immediately under an infra-red lamp for a short time and are then cut into strips which are placed in glass tubes containing a moisture-absorbing substance. We have found that the antibiotics in the strips are stabile for a long time (more than one year) when prepared and stored in this way.

It should be observed that, with this type of strips, it is possible to use any combination of antibiotics.

The strips have been tested in different ways in order to gain an idea of their fitness for use. The results obtained in the routine determination of bacterial sensitivity to chemotherapeutics will be published in two separate papers (Diding & Wallmark and Mollstedt).

Variations in inhibition zones for the same substance on different plates have been investigated in an experiment consisting of 40 plates. All these plates were prepared the same day with the same organisms (*Staph. aureus* 209-P and *Staph. albus*).

RESULTS

The results are presented in Table I, w gives the mean of the zones in mm.

The results show that variations in antibiotic concentrations in different s seem to be very slight. In our first ha made strips we had much larger variati

In order to ascertain the extent to w the inhibition zones vary when the strips used for a longer time, we have calcul the results from a number of assays mad different times. The values which ca

Table I. *Variations in inhibition zone the test strip method. Organisms: St aureus 209-P and Staph. albus. (40 assa*

Chemotherapeutic agents	Inhibition zones in r mean from 40 assa	
	Staph. aureus 209-P	Staph.
Sulfathiazole	9.2±0.16	0.7±(
Penicillin	10.7±0.11	4.0±(
Chlortetracycline	12.0±0.12	9.4±(
Oxytetracycline.....	12.6±0.14	10.6±(
Chloramphenicol	8.8±0.16	7.9±(
Streptomycin	12.0±0.10	12.5±(

Table II. *Variations in inhibition z by the test strip method. Organism: St aureus 209-P. (140 assays.)*

Chemotherapeutic agents	Inhibition zones mean from 140 ass Staph. aureus 209
Sulfathiazole	10.3±0.12
Penicillin	11.6±0.10
Chlortetracycline.....	12.2±0.10
Oxytetracycline.....	12.5±0.12
Chloramphenicol.....	8.3±0.11
Streptomycin	11.2±0.09

in Table II represent the mean from leterminations, 40 of which are those ble I, the other 100 having been taken routine determinations during two dif- periods. During these two periods eterminations were performed by dif- laboratory assistants.

e figures show, as may be readily under- that the means are different to those first experiment under these circum- es. Here there must also be varia- depending on small differences in , performance and reading of the results ifferent persons, and so on. The tance of these variations will be seen mparing Tables I and II. Variations s latter kind must, of course, influence sults of determinations made according paper disk method to the same degree. this reason, it seems to us to be of value to have a reference strain in- d in each determination. The results hen be based not on the absolute value inhibition zone which may vary from o day but on the relation between the for test and standard strain.

e strips have been used in the State riological Laboratory concurrently with the other methods used there, and we have found that the results conform well with those obtained with these methods.

SUMMARY

An agar diffusion method has been described for determination of bacterial sensitivity to chemotherapeutic agents by means of specially prepared paper strips. A short account of the method for preparing the strips is also given. — The method permits correction for many of the errors that impair diffusion methods. This is obtained by incorporating a referance bacterial strain with known resistance to the antibiotics in each assay.

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DETERMINATION OF THIOCYANATE IN SERUM WITH THE PYRIDINE-BENZIDINE REACTION

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(Received for publication June 7, 1955)

The physiology and biochemistry of the cyanogen compounds and the thiocyanates have lately acquired new interest. This is partly due to the importance of these compounds in connection with the regulating mechanisms of thyroid function and blood pressure, but is largely a result of newer aspects revealed in recent work on the chemistry of vitamin B_{12}.

In the body free cyanide ions are enzymatically converted to thiozyanate. As these two substances seem to be in equilibrium, a precise and specific method for the determination of thiocyanate in body fluids is required for further studies of their metabolism.

The methods so far employed for the determination of thiocyanate in biological material seem to have lacked either specificity or sensitivity. Boxer & Richards (1952) described a method which was reported to be both specific and sensitive. They oxidized thiocyanate to cyanide and determined it by their modification of the pyridine-pyrazolone method of Epstein (1947). However, the aeration technique involved complicates the method.

Aldridge (1944) has published a method for the determination of cyanide and thiocyanate based on conversion to CNBr in the presence of Br_2 and H_2O. Excess of bro is eliminated by means of arsenite, CNBr measured colorimetrically by orange red color developed in its rea with benzidine and pyridine in dilute tion. In some earlier work (1953) author has investigated the applicabili the pyridine-benzidine reaction to the mination of thiocyanate in blood serum. method was made sufficiently sensitiv determine the minute amounts of thi nates present in normal human blood se and it was shown that cyanide ions ma ignored as a source of error in s analyses.

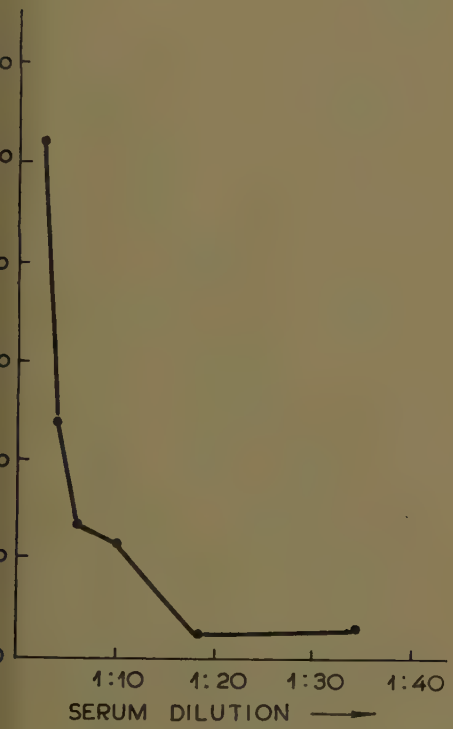
The present paper reports further fin on the accuracy and specificity of the dine-benzidine method as applied to s and describes a procedure for accurat termination of traces of thiocyanate serum.

EXPERIMENTS AND RESULTS

Important analytical errors were found caused by losses during deproteinization a non-specific color formation.

Deproteinization must be carried out acc to a special procedure in order to avoid co able loss caused by the partial binding of cyanate to the precipitated protein. Exper with deproteinization of the serum with

es of 10 per cent (w/v) trichloracetic acid recoveries showing losses of thiocyanate ging 12.3 per cent (Table I). The loss de- d with increased dilution of the serum with (Fig. 1) and was related to the concentration chloracetic acid in the liquid. Concentrations 10 per cent gave approximately 100 per cent ery of added thiocyanate. With lower con- tions, losses of thiocyanate were observed increased with falling concentrations of the itant (Fig. 2). It was found best to carry out eproteinization by diluting 1 volume of serum



The loss of thiocyanate following depro- tion of aqueous dilutions of one serum sample. OOH concentration: 3.3 per cent (w/v). ng for 10 minutes before filtration. KSCN : 2.0 ml 0.1 n solution per 2.0 ml serum. metric determinations with the ferric thio- cyanate reaction (Støa, 1952).

Table I. *The loss of thiocyanate in blood serum deproteinized with equal volume of 10 per cent trichloracetic acid in water. The measurements carried out according to the ferric thiocyanate method (Støa, 1952). Mean values based on series of 10 analyses.*

Sample	Observed values, mg SCN per 100 ml	Percentage recovery of added thiocyanate
Serum without addition	0.278±0.007*)	87.7±0.4*)
0.859 mg SCN added per 100 ml of serum	1.032±0.005*)	

*) Standard error of the mean.

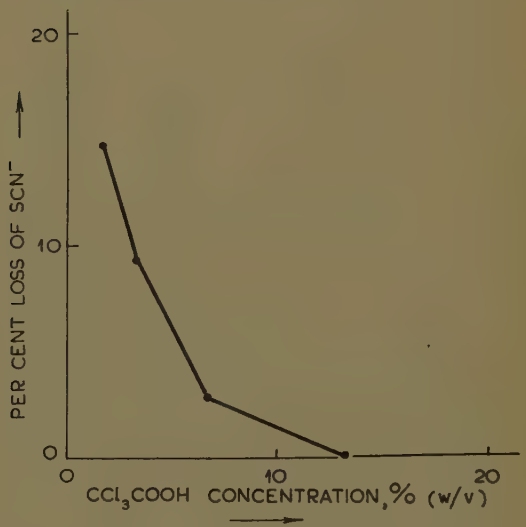


Fig. 2. The loss of thiocyanate in deproteinization of serum as related to the trichloracetic concentration. 2.0 ml 0.1 n KSCN solution and 2.0 ml trichloracetic acid solution added to 2.0 ml serum. Shaking for 10 minutes before filtration. Colorimetric determinations with the ferric thiocyanate reaction (Støa, 1952).

with 1 volume of distilled water and to precipitate with 1 volume of 50 per cent (w/v) trichloracetic acid. A check was carried out by the ferric thiocyanate method on serum with known amounts of added thiocyanate, and an average recovery of 98.7 per cent was found (Table II).

The pyridine-benzidine color reaction normally gives a certain amount of unspecific color when applied to biological fluids, as shown by increased absorption at shorter wavelengths. The interfering color is most pronounced for urine, but also considerable for serum. It is especially a source of error when the thiocyanate concentrations are low (Fig. 3).

In order to obtain a higher degree of specificity, experiments were carried out with precipitation of thiocyanate as its silver salt. Excess silver ions were removed by addition of sodium chloride, and the filtrate used as a blank. Addition of 1 vo of 1 N $AgNO_3$ to 5 volumes of deproteinized s filtrate was found to give quantitative remov the thiocyanate. The technique used is in prin as described for silver halides by Koltho Sandell (1948).

Table II. *Recovery experiments on one serum sample with known amounts of KSCN added. Serum diluted with equal volume of distilled water before deproteinization. 4.0 ml diluted serum precipitated with 2.0 ml 50 per cent (w/v) CCl_3COOH. Measurements according to the ferric thiocyanate method. Mean values based on series of 10 analyses.*

Sample	Extinction values (E 1 cm, 455 mμ)	Percentage recovery of added SCN
Serum without addition	0.023±0.000*)	
4.30 mg SCN added per 100 ml serum.....	0.319±0.002*)	98.7±0.3*)
4.30 mg SCN per 100 ml as aqueous KSCN	0.301±0.002*)	

*) Standard error of the mean.

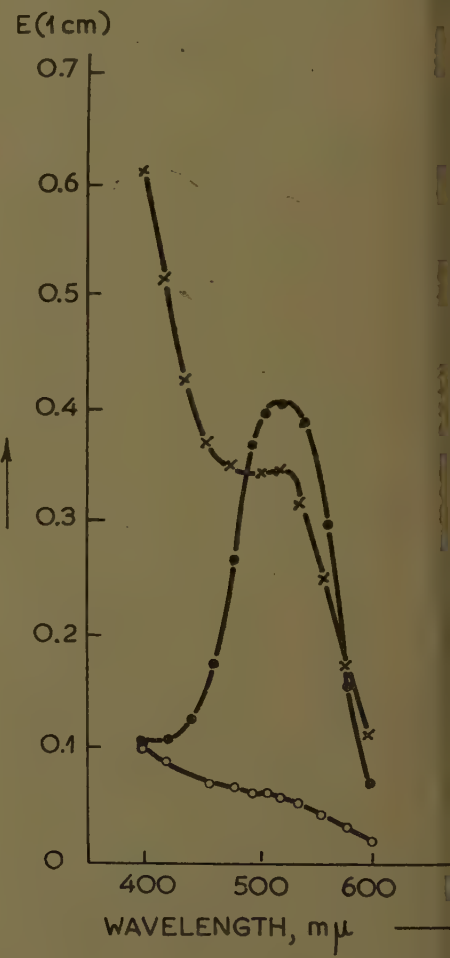
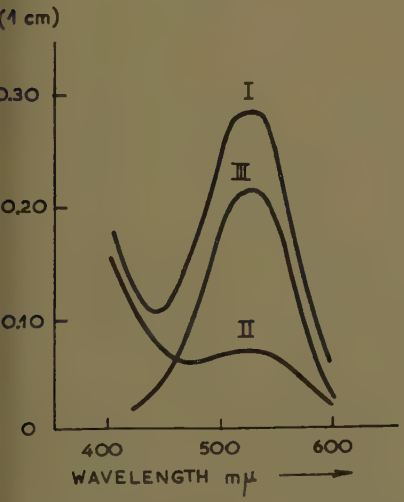


Fig. 3. Spectral absorption curves of the c solutions formed by the pyridine-benzidine re (1953) in biological fluids. x-x: Urine. Serum with SCN^- concentration above the a average. o-o: Serum with low SCN^- l

e scheme for the procedure finally adopted is lows:

DEPROTEINIZATION
↓
EPROTEINIZED SERUM
↓ + $AgNO_3$
FILTRATE free from
Cl^- and SCN^-
↓ + NaCl
FILTRATE free from Ag^+
↓
EST BLANK

e described procedure apparently makes pos- a specific determination of thiocyanate. ide ions, if present in the solution, will, how- give the same reaction. In figure 4 are plot- ne spectral curves of the reaction colors for ' and "blank", derived from a normal serum e. The measurements have been carried out st the usual reaction mixture with 1.0 ml H_2O



. Absorption curves of the colored solutions d by the pyridine-benzidine reaction in serum e. I: Untreated deproteinized serum filtrate st"). II: Deproteinized serum filtrate treated $AgNO_3$ and NaCl ("Blank"). III: Compensa- of unspecific color formation ("Test read st blank").

substituting 1.0 ml serum filtrate. Curve III illustrates measurements of the test against the "blank" and gives an expression of the specific thiocyanate color in the serum.

From Fig. 4 it will be seen that the spectrophotometric analysis of the color developed for the blank shows a small but distinct peak on the absorption curve at about 520 mμ. As it is well known that urea in aqueous solution exists in an equilibrium with cyanate, and thus might be expected to take part in the reaction, it seemed reasonable to investigate the possible significance of the substance as a factor in the color formation. In one experiment an aqueous solution of urea, 500 mg per 100 ml, was treated in the same way as the serum in preparation of the blank. The optical densities were read against a control solution produced in the same manner, but containing no urea. The spectral curve showing a small peak at about 530 mμ demonstrates that urea, if present in considerable amounts, may interfere to some extent with the color formation.

Analytical procedure

Reagents. Trichloracetic acid solution, 50 per cent (w/v) in H_2O.

$AgNO_3$, aqueous solution, 1 N.

Sodium chloride, analytical grade.

The bromine, arsenite and pyridine-benzidine reagents are prepared as described in the previous paper (1953).

Method. 2.0 ml of the serum is diluted with 2.0 ml of distilled water. 50 per cent (w/v) aqueous tricloracetic acid is added. The liquids are then mixed well, and filtered. The deproteinized filtrate thus prepared is kept for further steps. 1.0 ml of the filtrate is diluted with 0.2 ml distilled water. This solution is the "test". To 3.0 ml filtrate is added 0.6 ml $AgNO_3$ solution. The mixture is shaken well and heated for 5 minutes in a boiling water bath. It is then left for 1 hour to obtain complete precipitation, and filtered through a slow filter (Carl Schleicher & Schüll, No. 5893). A few grains of sodium chloride are added. The mixture is then shaken for about one minute, and the preci-

Table III. *Recovery experiments on different sera with known amounts of KSCN added.*

Sample	Observed values, mg SCN per 100 ml		
	Serum without addition	Serum with 0.527 mg SCN added per 100 ml	Percentage recovery of added SCN
1	0.313	0.848	101.5
2	0.305	0.844	102.3
3	0.313	0.854	102.7
4	0.310	0.861	104.6
5	0.308	0.843	101.5
6	0.276	0.797	98.9
7	0.271	0.798	100.0
8	0.269	0.802	101.1
9	0.282	0.809	100.0
10	0.265	0.797	100.9

pitate thrown down by 10 minutes centrifugation at 2000 r.p.m. The supernatant is the "blank". On 1.0 ml of test and blank respectively the color reaction is performed as earlier described (1953). The light absorption of the "test" is measured against that of the "blank" at 520 mμ.

Calculation of results. Using the above procedure, a series of measurements have been carried out on solutions of potassium thiocyanate, which were carefully checked by argentometric titration. The solutions were diluted with equal volumes of sodium chloride solution containing 1.2 g NaCl per 100 ml, providing a chloride content approximately equal to that of blood. Measurements on a Beckman spectrophotometer showed that the described modification of the pyridine-benzidine reaction follows the law of Beer-Lambert. For the calculation of the concentration of thiocyanate in the investigated fluid the following formula can be used:

$$\text{mg } SCN^- \text{ per } 100 \text{ ml} = \frac{E\ (1 \text{ cm } 520 \text{ m}\mu)}{0.89}$$

RECOVERY EXPERIMENTS

The method as described has been t on blood sera with known amounts of p sium thiocyanate added. The results of t experiments demonstrate the accuracy the method (Table III).

DISCUSSION

As stated above, the methods so far ployed for the determination of thiocya in biological fluids have several disad tages. In most of the methods the sensit has not been satisfactory, because the centrations of thiocyanate in most of body fluids under normal conditions relatively low. The pyridine-benzidine tion of Aldridge (1944) and the pyri pyrazolone reaction of Epstein (1947) possibilities for a considerably higher s tivity as compared with earlier meth Apart from the aeration technique of B & Richards (1952), all the methods hitl described seem to have lacked specificit least to some degree.

A very important cause of error in determination of thiocyanate in liquids taining proteins is the loss due to adsor of the anion. In the experiments report is shown that this loss can amount to 10 per cent. On the other hand, syste investigations show that the loss can be insignificant by diluting the fluid with prior to the precipitation with trichlora acid. Similar results have been obtaine De Mattos (1947). The concentratio trichloracetic acid during the precipitati also of importance because of the bindi thiocyanate to the proteins. It is shown the loss can be reduced to zero wit

ıg concentration of trichloracetic acid. case of serum, a concentration of 10 ent trichloracetic acid will give negli- eloss of thiocyanate ions, provided the is diluted with an equal volume of before the precipitant is added, and the ilution is 1 : 3.

ı previous paper (1953) it is concluded he pyridine-benzidine reaction gives ctory specificity in serum deproteinized richloracetic acid. On the other hand, s pointed out that a tendency to in- d light absorption at the vawelengths 400 mμ can frequently be observed, ıat some sera give an unspecific color nsiderable amounts. This unspecific can, in special cases, be so pronounced make exact measurements impossible. troduction of a "blank", in which the anate ions have been removed, a proce- is developed which seems to give a ic color formation.

stated above, the color developed for ank shows a small peak on the absorp- urve at about 520 mμ, corresponding e color formed by the thiocyanate or le ions. Considering the solubility pro- of the corresponding silver salts and common-ion effect" from excess Ag^+ (Kolthoff & Sandell, 1949) the con- tion of thiocyanate ions for a 0.9 per sodium chloridee solution in place of teinized serum, can be calculated as 10^{-8} mg per 100 ml. This should le the possibility of incomplete thio- te or cyanide precipitation.

e reported experiment on urea solu- seems to justify the conclusion that a n part of the color developed in the blank is due to the urea content of serum. However, with the urea concentrations in serum usually found, and even in uremia, the color formation may be regarded to be of no practical significance.

Another probable explanation regarding the observed peak is that traces of cyanide have been formed from amino acids during the bromination. Among others, Serin (1949) has pointed out that such reactions may take place under certain conditions. In any case, a cyanide formation during the procedure may be supposed to occur in "test" as well as in "blank", and accordingly it would be compensated for during the measurements.

Thiocyanate and cyanide ions can be determined in the same fluid by means of the aeration procedure as described by Aldridge (1944). As regards human blood sera, it is shown in an earlier paper (1953) that a content of CN^- ions usually can be neglected as a source of error.

SUMMARY

Considerable errors can occur in the determination of thiocyanate in blood serum if the deproteinization with trichloracetic acid is not carried out under certain conditions. The error is due to the binding of thiocyanate ions to the proteins. Minimum losses take place if 1 volume of serum is diluted with 1 volume of distilled water, and the proteins precipitated with 1 volume of 50 per cent (w/v) trichloracetic acid in water.

The pyridine-benzidine method, as so far employed, gives in certain cases considerable non-specific color, which makes exact measurements impossible. A "blank" is intro-

duced to counteract this disadvantage. Thiocyanate is precipitated as its silver salt and excess Ag^+ ions are removed with sodium chloride. Increased specifity is thereby obtained.

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THE EFFICIENCY OF DIGESTION IN GASTRECTOMIZED PATIENTS

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(Received for publication June 11, 1955)

e work here presented was undertaken
ne initiative of the late Professor of
ery Johan Holst. Since a long term
of the calcium metabolism in man was
d out in this laboratory, work on the
m balance in these patients was in-
d. It is reported in a following paper.
e of the problems of gastrectomized
nts is to maintain calorie balance. Gene-
xperience is that the food intake is low.
ever, the processes of digestion have
been subjected to a number of studies
somewhat variable results. The varia-
may be due to limited material, too
periods of observation, or to inherent
ences between the subjects studied.

far reports of a similar type have
red from the following:

ofman (1898), one nine day period in
atient.

oell, Losell & Karlmark (1927) one
day period in one patient.

rger & Konjetzny (1929) one twelve
period in one patient.

ima (1931) one 10—12 day period in
patients.

Bull & Stang (1934) one three day period in one patient.

Rekers (1943) several consecutive three day periods in three patients.

Farris (1943) one three day period in one patient.

Yugya (1951) one three day period in several patients.

The studies have been conducted shortly after the operation and at various intervals during the next four years.

Some have observed normal values for protein and fat digestion while others have found increased fecal output of nitrogen and fat.

Here is presented a study of the nitrogen balance and the fat absorption in 15 patients in whom total gastrectomy had been performed for carcinoma 3—43 months earlier. In addition a part of the pancreas had been removed in ten of the patients.

The material has been the subject of a study from other view points by Fretheim (1955).

EXPERIMENTAL

All the patients were called in from their homes to participate in the study. With the exception of one, all were out of bed and in relative well being. No signs of metastases were known at the time of the study.

Financial support received from New York unity Trust & Ely Lilly and Company.

Protein balance and fat absorption in gastrectomized patients.

Patient No.	Age & Sex	wt kg	No. of months after operation	Length of period days	Intake per day			Feces per day			N × 6 balan g
					Calories	protein g	fat g	protein g	fat g	% of dry	
10*	64 m	61	3	14	1380	53.0	72.3	2.9	10.1	60.5	+12
5*	49 m		4	14	1130	43.2	61.0	4.6	4.7	22.7	+ 4
16*	70 m	52	4	14	1520	43.8	80.8	5.2	15.4	58.9	— 5
18	66 m	58	7	11	900	35.8	49.0	7.8	12.1	42.4	—31
17*	63 m	53	8	17	840	30.3	46.2	6.8	10.6	37.2	—31
6*	51 f	42	9	18	1160	41.4	54.4	5.7	6.2	30.5	— 8
Average						41.3	60.6	5.5	9.9	42.0	—11
13*	60 f	53	15	14	1660	53.7	89.0	5.8	12.4	40.9	— 3
14	59 m	63	15	14	1400	40.2	83.2	3.5	4.4	30.3	—
2*	62 m	60	16	14	1790	73.0	79.0	20.7	13.7	26.6	+
12	59 f	52	20	14	1700	55.4	99.9	4.3	9.2	36.1	+
15	64 m		23	14	1470	41.8	87.6	6.3	8.2	27.6	—
Average						52.8	87.7	8.1	9.6	32.2	—
11	64 m	65	25	14	2100	67.6	124.7	6.7	7.3	30.8	+
7*	63 f	60	33	17	1210	45.3	62.2	4.0	6.0	39.8	+
8*	54 m	61	39	14	1480	45.5	71.4	6.1	7.4	31.7	—
9*	69 m	58	43	7	1370	32.1	69.8	6.9	9.1	41.6	—16
Average						47.6	82.0	5.9	7.5	36.0	—
Total average						46.8	75.4	6.5	9.1	37.2	—

*) pancreatic resection.

The diet was selected to be representative of a mixed diet. Its composition was made as simple as possible to economize with analytical work. Fillets of fish, lean meat, whole milk, cheese, potatoes, vegetables and fruits were used.

The food was weighed as served and ten per cent of it was sampled for analysis. Urine was collected in hydrochloric acid, feces was collected in vacuum pots and dried in vacuum every other day. The final dried feces was thoroughly powdered and representative aliquots taken for analysis.

The nitrogen was analysed by macro Kjeldahl and the fat by 20 hrs Soxleth extraction.

The daily calorie intake was low with an average of 1410 per day (range 840—2100).

RESULTS

The results are presented in the table

The figures have been arranged accor to the duration of the post-operative pe Only a few comments are needed.

The nitrogen balance was distinctly n tive in all except six. Since no proper trols are at hand on the same food in the nitrogen values for the feces mus judged in the light of general experie Only in one patient (No. 2) was the nitr excretion indicative of a greatly reduced

utilization. Considering the low food
ke, the values may be somewhat high in
e of the patients. The main impression,
ever, is that the negative nitrogen balance
iefly due to too low calorie intake. This
ention is strengthened by recent work in
tectomized dogs (McCorkle & Harper,
-), in which increased nitrogen loss in
feces was observed. Nitrogen balance
however, achieved with increased food
ke in most of the 120 totally gastrectom-
dogs.

he figures for fecal fat undoubtedly in-
te a reduced fat absorption. On the other
l on an average nearly 88 per cent of
ingested fat was absorbed.

he results thus confirm largely what a
ber of earlier workers have observed.
n the point of view of energy balance
the main problem appears to induce the patients to eat enough food. The excess fat in the feces and the comparatively low intake of fat-soluble vitamins in such patients would indicate the need for extra vitamin intake. Surely such individuals might benefit from the regular use of fat soluble vitamins in concentrated form.

SUMMARY

Nitrogen balance and fat absorption were studied in 15 gastrectomized patients in the 4th—44th month after operation. A very high N output in the feces was observed in one patient only. The fat content of the feces was increased in practically all. The nitrogen balance was negative in all except six, indicating a too low intake of calories.

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PHOTO-ACTIVATION AS A SOURCE OF ERROR IN THE CEPHALIN CHOLESTEROL FLOCCULATION TE

By O. BASSIR and J. HALL

From the Biochemistry Laboratory, Westwood Hospital, Beverley, Yorks, Great Britain

(Received for publication April 20, 1955)

The dynamics of the mechanism of action of Hanger's (1939) cephalin cholesterol test of liver function has not been completely elucidated. Pohle & Stewart (1941) obtained positive results with the test in 18 out of 23 cases of obstructive jaundice and 56 out of 58 patients with parenchymatous dysfunction of the liver. Lawler and Hirst (1950) correlated the cephalin cholesterol reaction of the blood serum with the post mortem diagnosis of hepato-biliary disease in 122 cases, and obtained satisfactory agreement only in toxic hepatitis (75 per cent positive). Neefe & Reinhold (1944) experiment with sera of 13 normal persons and 7 patients with liver disease and showed that expo of the serum-antigen suspension to br light increased the flocculation. They gested that photosensitivity was the c of the discrepancy often observed when same test was carried out with the reagents in different laboratories. T workers observed that the reaction in dued light and total darkness was usuall same.

EXPERIMENTAL PROCEDURE

324 specimens of blood serum from hos patients were subjected to our routine cep cholesterol test (a) in darkness, and (b) in diff light. The serum-saline-antigen mixtures wer

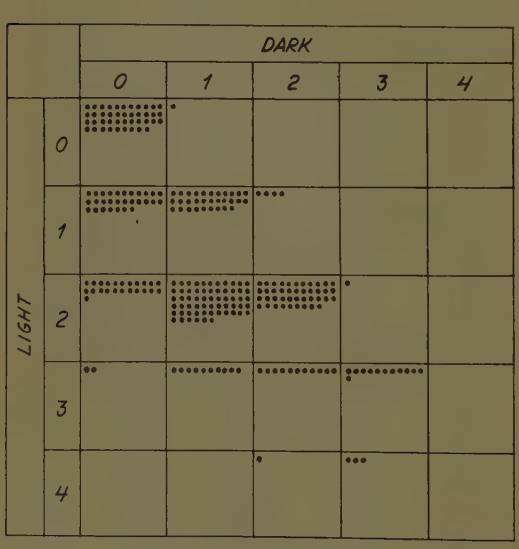


Fig. 1. Correlation of light and dark readings at 24 hours.

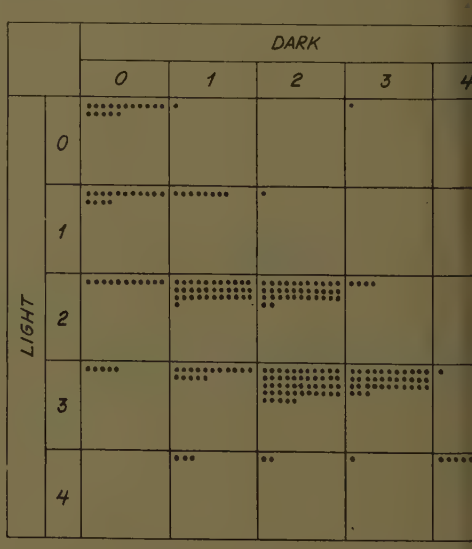
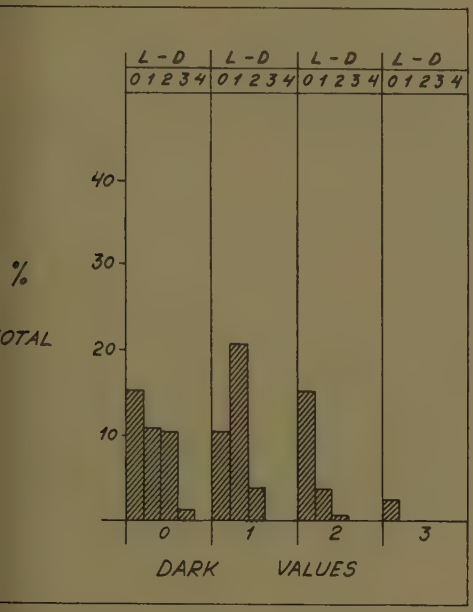


Fig. 2. Correlation of light and dark readi at 48 hours.



3. Correlation of light and dark readings among the expected negatives at 24 hours.

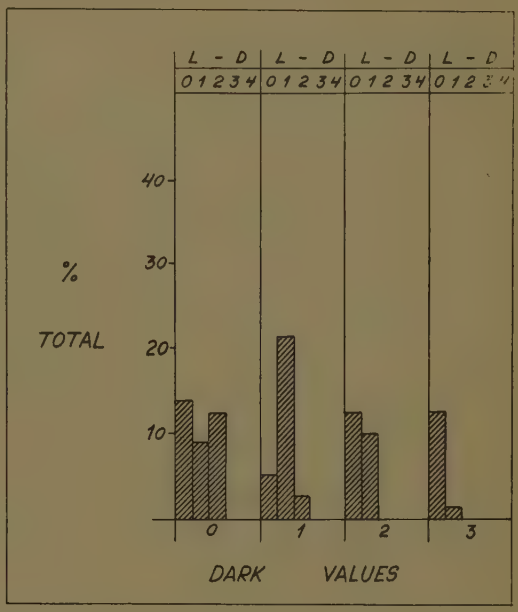


Fig. 4. Distribution of the light and dark readings of the expected positive at 24 hours.

ling in stoppered glass tubes, and flocculation ngs were taken after 24 hours and 48 hours. rol tubes were set up simultaneously. The ts were recorded in the following units: no le change = 0; the slightest change detectable complete flocculation with a clear supernatent nearer to 1 than 4 = 2; nearer to 4 than 3. The cephalin cholesterol antigen was ob- d from DIFCO and prepared form the same of reeagent, as directed by the makers. The ts obtained with specimens exposed to light lesignated L, and the others D.

RESULTS

Figures 1 and 2 give the correlation of Light and Dark readings at 24 hours 48 hours, respectively. When D = 0.1, respectively, the percentage of instances which L — D > 0 are 56, 69, 21, 24 at ours; and 66, 85, 59, 3 at 48 hours. It be seen that the influence of light is most ked in the region of least visible floccula-

he sera studied could be classified into groups: (i) those from patients suffering hepato-biliary disease, (ii) those from patients with no clinical evidence of liver dysfunction. Positive flocculation may be expected from group (i) but not from group (ii) (Hanger, 1939). Figures 3 and 4 show our results analysed in this way at 24 and 48 hours respectively. The figures suggest that the photosensitivity of the two kinds of

	24 Hour Reading							
	0		1		2		3	
48Hr-24Hr	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
-1								2
0	18	20	17	9	10	13	2	5
1	12	9	21	18	10	11	1	7
2	6	2	3	4				

Fig. 5. Distribution of differences of dark readings at 24 and 48 hours, as percentage of total tests.

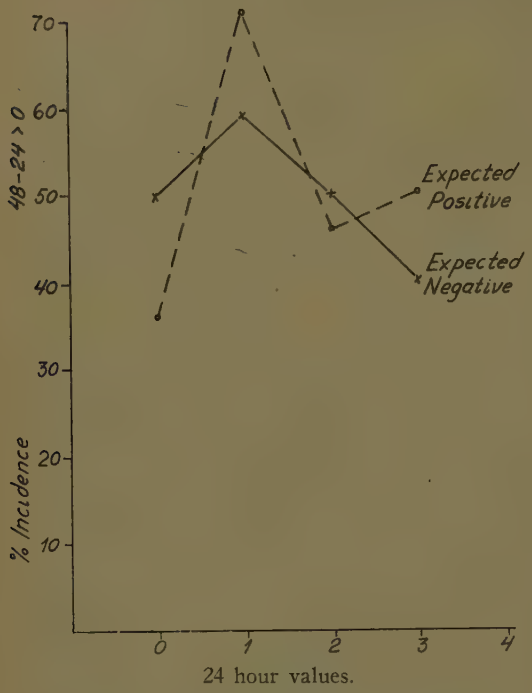


Fig. 6. Variation in dark readings at 24 and 48 hours.

blood serum is of approximately the same order, and not the main cause of the insensitiveness of the test, with regard to differential diagnosis.

The last inference is supported by Figures 5 and 6 in which the results of the dark experiments are correlated at 24 and 48 hours.

DISCUSSION

The experiments described in this paper confirm that photoactivation is a major factor introducing error in the cephalin cholesterol liver function test. The influenc is towards greater flocculation, and could therefore derive from the energy-transfer of light particles. However, a detailed consideration of the possible dynamics of such a process would be out of place here.

Since Hanger's (1939) cephalin cholest reaction is sometimes used as a scree device in suspected liver conditions, experimental results described above highly significant. It is clear from Figu that while the test is unlikely to be negati cases of hepato-biliary disease (where p tive results are expected) it is positiv such a high percentage of non-hepatic ditions as to be unreliable as a scree procedure.

Furthermore, it seems unlikely that pathological character of the serum is rel to its photosensitivity to the cephalin-c esterolantigen. It might be profitable t vestigate the physico-chemical react which constitute the process of 'ripenin the cephalin cholesterol suspension fro time of preparation to when it is ally used.

SUMMARY

1. It has been shown, with 324 specim of blood serum from hospital patients, the cephalin cholesterol flocculation is a erated by exposure to diffused light.

2. The blood of patients with liver dis is not significantly more photosensitive that from other persons.

3. The low value of the test, as a scree agent for liver function, is placed on a q tiative basis.

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PHOSPHOLIPID DETECTION IN PAPER ELECTROPHORESIS*

By B. A. SACHS and E. DANIELSON

From the Medical Division, Montefiore Hospital, New York City, U.S.A.

(Received for publication June 6, 1955)

ıe cytochemical technique of controlled mation, as described by Elftman (1954) he staining of phospholipids in tissues, been modified for paper electrophoresis. method utilizes dichromate oxidation rigid control of the critical variables H, time, temperature and concentration. rolled chromation has made possible the atoxylin staining of phospholipid after r electrophoresis without affecting the r lipid constituents of the serum.

arly in this study it was found that the odified histochemical technique stained f the protein bands of serum. Such ing occurred independently of the pre- e of lipid ,as was shown when serum ein from which all of the lipid had been acted with modified Bloor's reagent olute alcohol, 3 parts, anhydrous ether, rt) also stained with hematoxylin. To onstrate phospholipid it was therefore ssary first to remove the protein from trips. This was accomplished by trypsin tion.

oncentrated phospholipid in the form of bean lecithin dissolved in chloroform and bean lecithin dissolved in soy bean oil being streaked on filter paper stained ep black with the staining technique ibed below. Lecithin in the concentra- found in normal human serum stained gray. The following substances when streaked on filter paper did *not* stain:

olive oil	oleic acid
soy bean oil	palmitic acid
peanut oil	stearic acid
glycerol trioleate	cholesterol
glycerol tributyrate	glycerol phosphate
	phenol phosphate

Cod liver oil did stain but was rich in phospholipid by chemical analysis.

Serum phospholipids appear as a dark gray band at the β-globulin region and as a faint gray band at the α-1 globulin-albumin region. The areas stained coincide with the position of phospholipid recovered after elution of paper electrophoretic strips by Swahn (1953). Comparison of this stain for phospholipid with Durrum's oil red 0 stain for lipid (Durrum, Paul & Smith, 1952) revealed that the phospholipid, in most instances, stains an area which corresponds to the β-lipoprotein band. In hyperlipemia, this band overlaps into the "neutral fat" zone which lies between β- and γ-globulin.

The intensity of the stain has been found to be directly proportional to the amount of chemically determined lipid phosphorus in the serum and varies from a very faint gray in serum with a low normal phospholipid to black in sera with hyperphospholipidemia. The intensity of the bands may be increased by shortening the time of electrophoresis. This decreases the total area of migration and makes each band less diffuse (Fig. 1).

The method for phospholipid detection on filter paper follows:

This study was supported in part by grants the Lasdon Foundation, the National Institute thritis and Metabolic Diseases of the United Public Health Service and from the Medical rch and Development Board of the Surgeon al's Office.

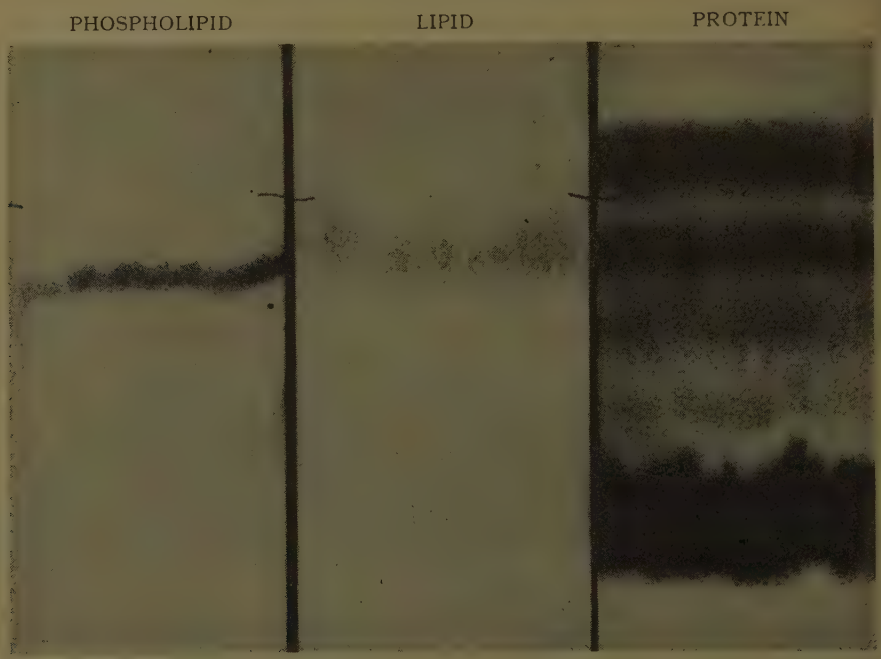

Fig. 1. Phospholipid-lipid-protein interrelationships. Paper electrophoresis was performed in a m fication of Durrum's apparatus (Sachs, Cady & Ross, 1954). The strip was stained for pro with naphthalene black 12 B 200, for lipid with oil red 0 and for phospholipid with the method he described. The lipid stain is red and not as intense in the black and white photograph as in the ginal. Chemically determined lipid phosphorus in this serum was 12.2 mg per cent.

Reagents

1. M/5 acetate buffer containing 0.2 M acetic acid and 0.2 M sodium acetate.
2. 2.5 per cent potassium dichromate, adjusted to pH 3.5 with acetate buffer.
3. Freshly prepared 0.1 per cent hematoxylin containing 0.005 per cent potassium ferricyanide adjusted to pH 3.0.
4. Trypsin, crystalline. As supplied (Armour Tryptar) 30 ml of buffered trypsin contains 250 mg of tryptic activity. This was diluted to 100 ml with 0.9 per cent saline. Greater concentrations of trypsin may be used with hyperproteinemic sera.

Method

1. Following electrophoresis, place dried filter paper strip in 50 ml of trypsin solution (containing 125 mg of tryptic activity).
2. Incubate for 3 hours at 37° C.
3. Air dry strip.
4. Place strip in potassium dichromate solution at 50° C for 18 hours.
5. Wash strip with distilled water repeatedly yellow dichromate is completely removed.
6. Stain with hemotoxylin solution for 2 at 56° C.
7. Wash with distilled water as in 5.
8. Air dry.

CONCLUSION

Controlled chromation, as describe Elftman (1954), has been modified for pholipid detection on filter paper after trophoresis.

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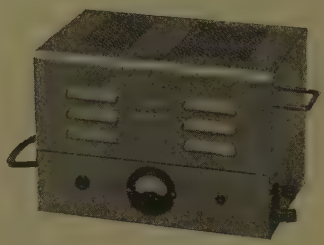

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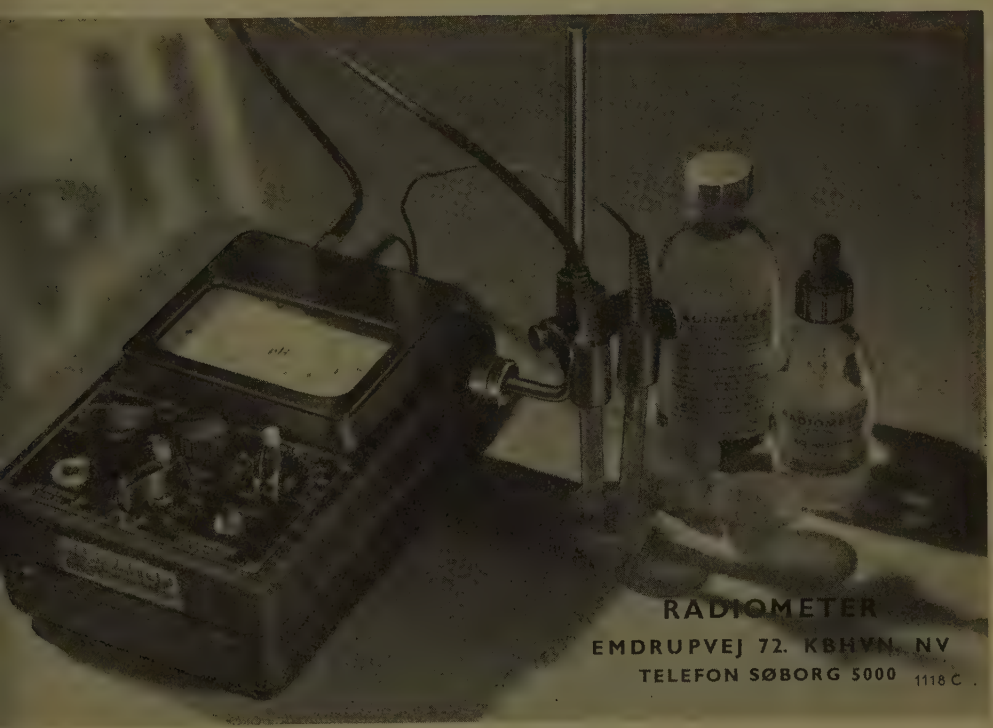

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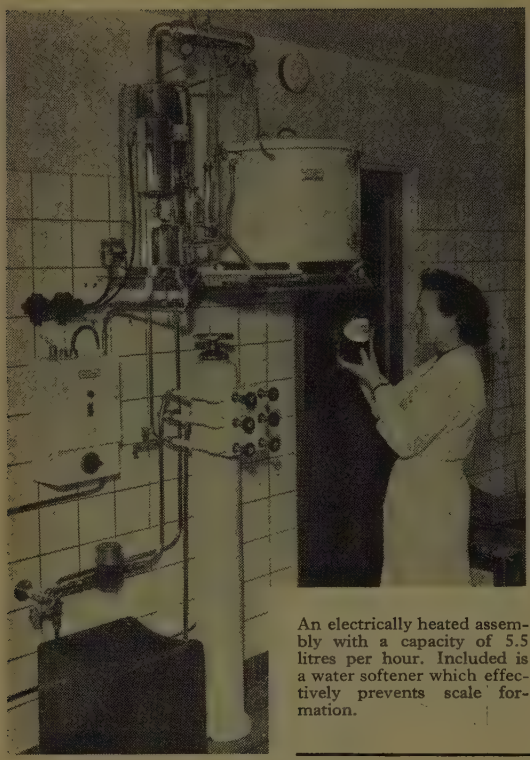

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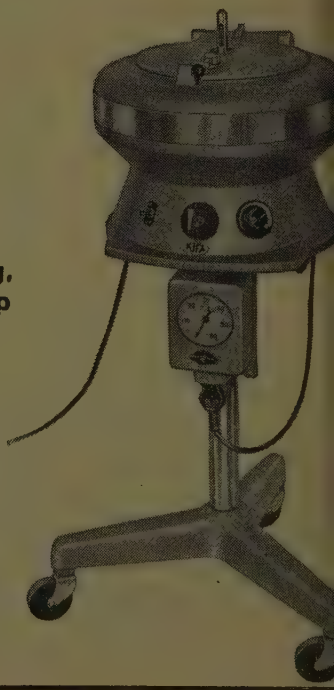

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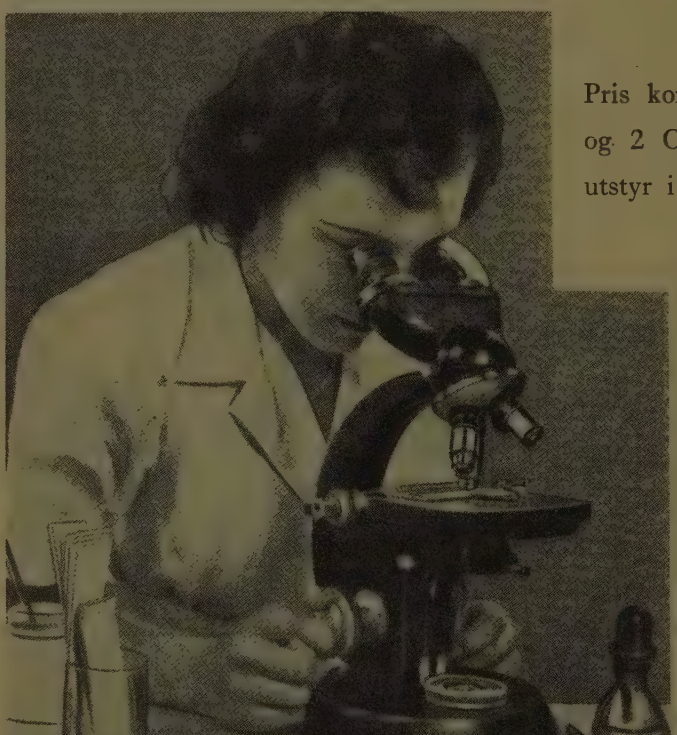

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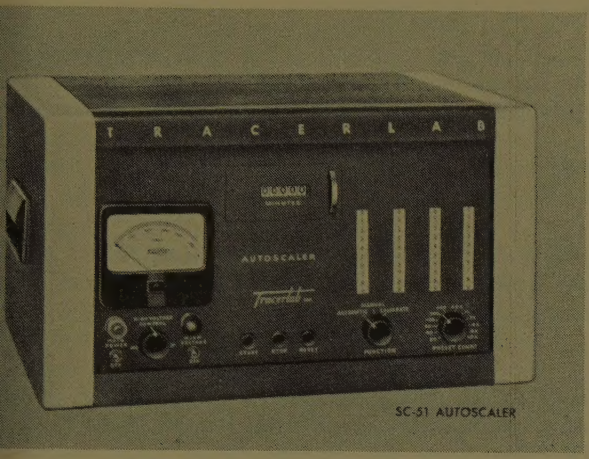

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